THE NATURE OF THE ACTION POTENTIALS OF CRUSTACEAN MUSCLES

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(With Plates 7 and 8)

INTRODUCTION

The muscle-action potentials from decapod crustaceans have been recorded many times by different investigators. These observations have established the fact that these potentials differ considerably from the action potentials of vertebrate striated muscles. Attention has been mainly focused on the phenomena of facilitation (Wiersma, 1933; Katz, 1936; van Harreveld & Wiersma, 1936; Wiersma & van Harreveld, 1938), while much less notice has been taken of the electrical sign of these potentials. Biedermann (1888) observed that in the adductor muscle of the claw of *Astacus* the electrode placed in the muscle substance became positive relative to one placed distal to the tendon of this muscle. This observation has since received special attention only by Serkoff (1935). Biedermann's explanation was that the electrode in the muscle functioned as the indifferent one under the circumstances, since the hole made for it would cause so much damage that no activity would take place in its neighbourhood. By cutting the distal part of the muscle and applying an indifferent electrode to this cut, he found that the lead in the body of the muscle then became negative. Serkoff observed that a small injured region near the end of the muscle could become even more electro-negative during excitation than during rest and therefore doubted Biedermann's explanation. He came to the conclusion that the potentials obtained did not arise under the electrodes but some distance away from them, and that the distal parts became more negative than the proximal ones owing to the peculiar anatomical structure of the adductor muscle. The cause of this asymmetrical distribution of potential is not discussed. During the course of our investigations it became clear that unexpected signs of the action potentials are the rule for crustacean muscles, and in these cases the explanations provided by the papers cited above were inadequate.

METHODS

The action potentials from muscles of *Cambarus clarkii* were used throughout this investigation. They were recorded with the aid of a cathode-ray oscillograph connected to the preparation through a high-gain differential amplifier. In all cases the muscles were stimulated indirectly by square-wave electric pulses which also

activated the sweep circuit of the oscillograph. Various types of leading-off electrodes were used; generally platinum wires wrapped in a thin layer of cotton soaked in the physiological solution described by van Harreveld (1936) were employed. The muscles investigated included the closer (adductor) and opener (abductor) of the claw, the extensor of the carpopodite of the cheliped, the first medial segment of the anterior thoracic muscle and the posterior adductor muscle of the mandible.

RESULTS

Closer of the claw. To exclude the possibility that muscle damage would be a factor in the results obtained, a series of experiments was performed in which the electrodes were attached to the outside of the shell of the propodite in various positions. It was found that the potentials obtained in this way were smaller but otherwise similar to those obtained from the same placements when holes were subsequently made. Therefore the slight damage to the muscle caused by insertion of the lead wire is insignificant as far as the electrical sign of the action potentials is concerned, confirming Serkoff's (1935) finding that a small wound does not completely inactivate the surrounding tissue as would be required for Biedermann's (1888) explanation of the positive-action potentials.

In further confirmation of Serkoff it was found that an electrical gradient existed along the long axis of the muscle during activity. The distal part of the shell became more negative than the proximal part, and this relationship held true for any given electrode distance. The deflexion obtained was mainly monophasic though often complicated by smaller variations.

The potentials thus far described were obtained from the muscle on stimulation by way of the fast-closer nerve fibre, resulting in twitch contractions. The potentials recorded when the muscle was stimulated by means of the isolated slow-closer nerve fibre followed the same rules of polarity, but were smaller in size. These show, of course, in contrast with the fast-twitch action potential, the facilitation previously described by van Harreveld & Wiersma (1936).

Opener of the claw. The action potentials of the opener muscle are known to resemble those of the slow closer in size and shape (Marmont & Wiersma, 1938). When led off through the intact shell from the region about the muscle, results similar to those from the closer were obtained. Again the muscle potentials were preponderantly monophasic and again the distal regions became more electronegative than the proximal ones. The structure of this muscle is much simpler than that of the closer in that the muscle fibres are more restricted to one plane. It therefore follows that the complex structure of the closer muscle has no significance in the peculiarity of the electrical sign of its action potential.

Anterior thoracic muscle. For further study of the action potentials it seemed desirable to exclude the influence of the feather-like arrangement of the muscle fibres. Therefore the next preparation used was that of the first medial segment of the anterior thoracic muscle. After evisceration there is found on the ventral side of the thoracic cavity a long muscle, divided into parts by tendinous tissue, each segment of the thorax from the second being represented by one such division.

The first of these divisions inserts on a ridge of chitin and is clearly split into two parts, a medial and a lateral, which join in the second segment. The muscle fibres in both these parts run nearly parallel. As shown by Wiersma (1938) the whole thoracic muscle can be brought into practically simultaneous action by stimulation of the giant fibres in the central nervous system. These fibres are in this preparation easily accessible in the oesophageal commissures.

It was always found that an electrode placed anywhere on the dorsal surface of this muscle, which is completely exposed on evisceration, became electro-positive relative to an electrode placed on inactive tissue regardless of the inter-electrode distance. Since other parts of the thoracic muscle as well as muscles in the neighbourhood attaching to the legs also contract on stimulation of the giant fibres, further analysis proved difficult (see, however, below). The potentials obtained were large, primarily monophasic spikes, but complicated by numerous small variations attributable to the activity of these surrounding muscles. However, the conclusion is warranted that the feather-like arrangement of fibres in other muscles is not the factor responsible for the positivity of their muscle action potentials.

Posterior adductor of the mandible. We turned our attention to the mandible muscle since it is the only large muscle in the crayfish which can be approached easily from all sides and on which electrodes can be placed on any part of its surface (see Pl. 7). It was found that this muscle responds with a twitch contraction on indirect stimulation with a single shock. The nerve runs along the tendon and follows it in its ventro-dorsal course. Preparation of the nerve is difficult but not necessary, as very good results were obtained by placing the stimulating electrodes on the caudal side of the tendon thereby contacting the nerve. The muscle was isolated by cutting away a curved rectangular piece of carapace, including both the origin on the carapace and the insertion on the mandible.

The muscle may be best described as a half-cone, the flat side being the lateral (carapace) side, and the fibres which are the continuation of the tendon meet the carapace at almost a right angle, whereas the fibres on the medial side form a distinctly sharper angle, which diminishes noticeably during contraction.

When the action potentials of this preparation were recorded by placing one electrode at the middle of the muscle and the other on the tendon, the body of the muscle became electropositive with regard to the tendon lead. It thus seemed inevitable that active crustacean muscle tissue became positive with respect to inactive tissue. Careful probing with the electrode on the muscle disclosed, however, that though most of the muscle surface gave positive potentials, one small area became electro-negative during activity. This area was located very near to the carapace on the lateral flat side of the half-cone and registered a negative spike of about 12 mV. compared to the positive spike of 12 mV. recorded from the opposite curved side of the cone (see Pl. 7). When the electrode approaches this negative area the positive spike first diminishes in amplitude, then becomes negative after passing through a stage in which the amplitude is small and the deflexion diphasic.

When a small cut was made in this negative area its action potential immediately became about as strongly positive as it had been negative. Artificially inactivating

other areas on the muscle resulted in a further increase of the original electro-positivity. These experiments indicated that the tendon lead became actually more negative during activity than most other parts of the muscle surface. At first glance it was not obvious how this was possible. The only other connexion of the tendon with other parts of the muscle was through the shell, as it curved from the origin of the muscle to the tendon, and it was considered that the electrical resistance of this pathway would be much greater than that of the wet muscle surface itself. If this were the case it ought not to have much influence on the electrical recording. However, further isolation by cutting the shell between the origin and the tendon resulted in a striking effect: all muscle action potentials were from that time on negative, the largest arising from the small area which showed originally the electro-negativity. By replacing the connexion between the carapace and tendon by means of a piece of cotton-wool soaked in physiological solution the original positive potentials were restored and the sign of the potentials could in this manner be changed at will. Therefore with the shell intact, or with the cotton connexion, the tendon lead is the more electrically active, but becomes the less active when these connexions are broken.

Not only the whole muscle but also each fibre is conical in shape. This may account for the increase in the size of the action potentials observed when, using the completely isolated preparation, the electrode is placed closer to the carapace, where the thicker ends of the fibres are situated.

Extensor of the carpopodite. The inner surface of the extensor muscle can be rather easily exposed without damage by carefully removing the flexor muscles in the meropodite and the part of the shell to which these are attached. Although this muscle lacks the advantage of being approachable from all sides, as in the case of the mandible muscle, most of its fibres are cylindrically shaped. Furthermore, it has the feature that the ramification of its nerve supply takes place on the exposed side (van Harreveld, 1939). Thus the three nerve fibres supplying this muscle can be lifted on micromanipulated electrodes and their branches cut, if desired. The muscle fibres run in feather-like arrangement from the tendon to the shell, and it is possible to probe the exposed surface of a single muscle fibre with a micro-manipulated lead.

It was found that with single shocks these fibres gave large monophasic negative spikes relative to the solution in which the ischiopodite was dipped. These potentials diminished in size when the probing electrode was placed near the tendon or near the shell and reversed to positive in many instances on the inactive insertion of the fibre. The shell was positive with respect to the solution as were potentials obtained from the outer surface of the muscle by leads through holes in the shell.

When a part of the muscle was denervated (see Pl. 8) by cutting a small branch of the ramification of the nerve fibres as indicated in the figure by the arrow, the exposed surface of this part (position 1) then became electro-positive relative to the solution during activity. By moving the electrode to the first contracting fibre (position 2) of the innervated area a reversal was immediately obtained, and there was thus a very sharp electrical boundary between these two parts, active

and inactive, of the muscle. These experiments showed that in certain muscles it is indeed possible to lead off negative potentials from the exposed surface of muscle fibres; therefore exposure itself is not the reason why in other cases the surface does not become negative.

The nature of the action potentials. In previous publications (see Wiersma, 1941) there have been presented different arguments for the local nature of the crustacean muscle action potentials. Our present experiments are in complete accord with these views. If an action-potential wave were started at one spot on the muscle fibre and conducted from there over the muscle-fibre surface one would expect that with two leads on the fibre diphasic potentials would result. Furthermore, with the leads placed oppositely across the muscle both electrodes should become equally negative at the same time and no potential difference between them should be recorded. However, monophasic potentials were always recorded when the leads were placed on the same side of a muscle fibre in the extensor muscle and cross-sectional potentials were, as stated, easily observed in the mandible muscle by placing one electrode on the lateral, flat side and the other on the curved one. In order to confirm this last point on a muscle with parallel fibres, the thoracic muscle was once more turned to. It was found possible to free completely the muscle from all surrounding tissue except the anterior end attachment, though this separation leads to damage of the muscle parts which attach to the thorax wall posteriorly. A sufficiently long section of the nerve innervating the anterior segments from below can be isolated and makes indirect stimulation of this preparation possible. In this way negative monophasic action potentials were obtained from both the under and the upper sides of the muscle against an indifferent electrode, placed either on the posterior inactive part of the muscle or on the insertion. The largest deflexions resulted with the active electrode on the under side. Because of this difference in potential from the two sides it was clear that cross-sectional leading off from this very thin muscle would give a noticeable potential difference. Indeed, this was found to be the case. Even when the electrodes were brought exactly opposite one another and thus were separated by a muscle fibre layer certainly less than 2 mm. thick, a significant deflexion was obtained. Control experiments, using the frog sartorius, gave under the same condition no such cross-section potentials, illustrating the great difference between these two parallel fibred muscles.

Obviously the action potentials recorded from the crustacean muscles are the resultant of the difference in voltage of two spikes from two places, which are not necessarily the places where the electrodes make contact with the tissue. The nerve conduction time is an important factor in determining the shape of the recorded deflexion, and diphasic action potentials are observed when the muscle areas, whose action is registered, are innervated by nerve branches of sufficient difference in length to cause a noticeable time interval between the start of their activities. This explains, for example, the diphasic potentials led off from the closer muscle.

We have, thus far, not succeeded in mapping out the finer potential distribution of a single muscle fibre during contraction. If the muscle potential is really a series of local potentials as our hypothesis asks for, it would be expected that a fibre

would be more negative in the neighbourhood of the nerve endings than in the intervening spaces. Careful probing with platinum micro-electrodes of 0·01 mm. diameter failed to reveal any constant differences.

DISCUSSION

From the data presented it is clear that, when the active tissue can be sufficiently isolated to insure against any possible extraneous connexions between it and the indifferent lead, the action potentials obtained are negative. This does not explain why monophasic positive potentials consistently appear with many different electrode placements. It is our contention that several factors contribute to this phenomenon, the most important of which is the nature of the action potential. If conduction of the action potential took place, then the summation or subtraction of the voltages observed could not occur, and instead of the monophasic, diphasic action potentials should result under these experimental conditions. In previous publications (see Wiersma, 1941) other arguments have been offered why conduction of excitation over the crustacean muscle fibre is by nerve fibre conduction only. Therefore the following hypothesis in which all muscle potentials are considered to be of local origin is proposed.

Crayfish muscle fibres receive their innervation mainly on one side; this innervation, in contrast to the end-plate formation in one region as present in vertebrate striated muscle fibres, consists of a profuse branching with many endings contacting the muscle fibre on this side. Each one of these many endings upon reception of a nerve impulse causes the immediate surrounding muscle fibre area to depolarize and therefore this whole side becomes negative more or less all over during activity, though intermediate small areas of relatively less negativity between separate nerve endings should be present. The other half of the muscle-fibre surface is either without or at any rate with considerably fewer innervated points and thus becomes overall much less negative. In adjoining muscle fibres the orientation of the innervated and the non-innervated side is usually the same.

Considering this hypothesis, if the local nature of the action potentials is accepted, it follows that, in order to record any potential whatsoever, not all of the muscle fibre can become equally negative; each fibre must somehow provide both source and sink in order that an electric current and voltage exist. In the case of the local or conducted potentials in nerve or other excitable tissues, the depolarization is definitely confined to one small area which acts as a sink, the surrounding polarized region providing the source of the current.

If the crustacean muscle fibre showed an equal distribution of its local potentials by equal distribution of the nerve endings or by a series of rings of negative potential (like the cross-striations), it would be possible to obtain action potentials from them only by probing the fibre with two micro-electrodes and thus locating areas of slightly different potential. No measurable change would exist at any distance. In order to record potential differences such as do appear there must be electrical asymmetry which is possible if crustacean muscle fibres are unilaterally innervated. In that case the fibre itself acts as a small cylindrical battery with the negative pole

the innervated side and the positive pole the non- or only slightly innervated side. According to this hypothesis the crustacean muscle fibre would be comparable to a single electroplax of the electric organ in certain fishes (Cox, 1943).

The evidence for the existence of one-sided innervation is at the present time rather indirect. van Harreveld (1939) found innervation on one side only with silver staining; however, he believed that the other side would have a similar number of nerve endings, which had remained unstained. It was found during the present investigation that with methylene blue staining, which does not generally show the finer endings of the sublemnal branches, but which often demonstrates the many places where the nerve branches become sublemnal, these places always appear only on one side of the muscle fibre. In the thoracic muscle the innervation clearly branches on the underside of the muscle and no large branches are present on the upper side, though in the most anterior part the main nerve branches are no longer on the underside itself, but run in the body of the muscle between the fibre layers. The latter situation has also been found in a number of other muscles, and in these cases it is possible that part of the muscle fibres receive their innervation from one side and the other part from the opposite side.

It seems highly significant that negative action potentials are obtained from the muscle surface on the side from which the muscle is innervated. Thus the extensor muscle receives its innervation from the inside and becomes negative on this side; the anterior thoracic muscle is, as stated, innervated mainly from the underside from which the largest negative spikes are obtained when this muscle is isolated from the surrounding tissue. In further verification of this rule the lateral side from which the nerve enters the mandible muscle is the side on which the negative area is located.

Since we have shown crayfish muscle fibres to be relatively inactive near the insertion end (see Results—Extensor) and yet find that the carapace to which the mandible muscle is attached is more negative than most of the surface of the muscle itself, it follows that the orientation of the innervated sides of the muscle fibres to the shell is of greater consequence than the inactivity of the muscle endings. This same factor accounts for the potential gradient over the claw where near the carpopodite the shell contacts the inactive ends of the fibres at nearly right angles, whereas towards the tip the shell is exposed to the lateral sides of the fibres only.

It is not clear at present why the carapace acts as a better electrical connexion between the body of the mandible muscle and the tendon than does the muscle substance itself. Further experiments are planned to elucidate this question.

SUMMARY

An investigation has been made of the action potentials of the muscles of the crayfish, *Cambarus clarkii*. It was found that the polarity of these potentials was in many instances the reverse of that expected.

It is shown that 'positive' potentials cannot be explained by injury to the active tissue, by the complex structure of the muscles, or by the direction of the muscle fibres.

Using muscles in which all extraneous connexions could be rigorously avoided, the muscle action potentials were invariably found to be negative.

Direct evidence is presented for the local non-conducted nature of the crustacean muscle action potentials. An hypothesis is offered to explain the monophasicity and polarity of these action potentials on the basis of multiple unilateral innervation of the muscle fibres.

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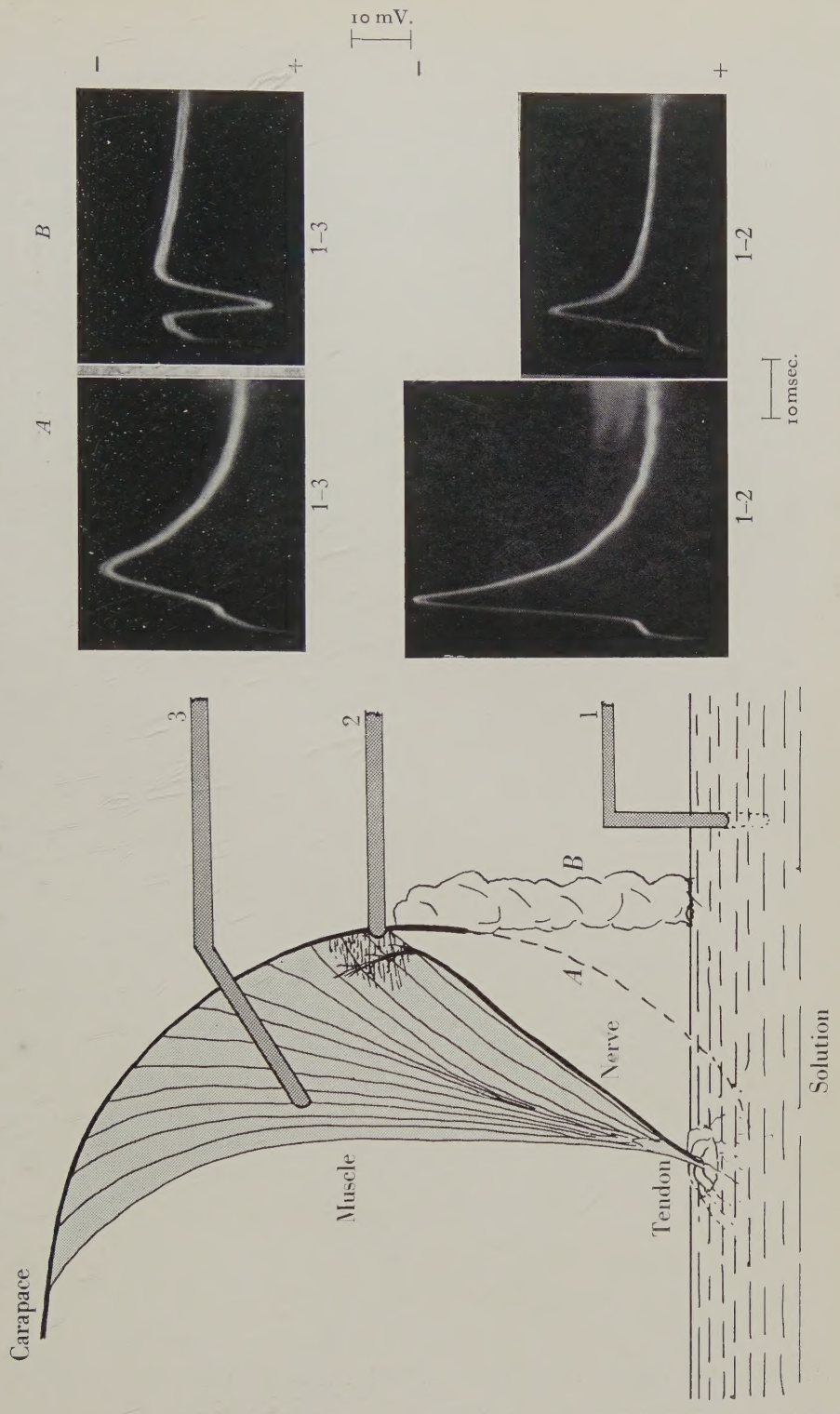
EXPLANATION OF PLATES

PLATE 7

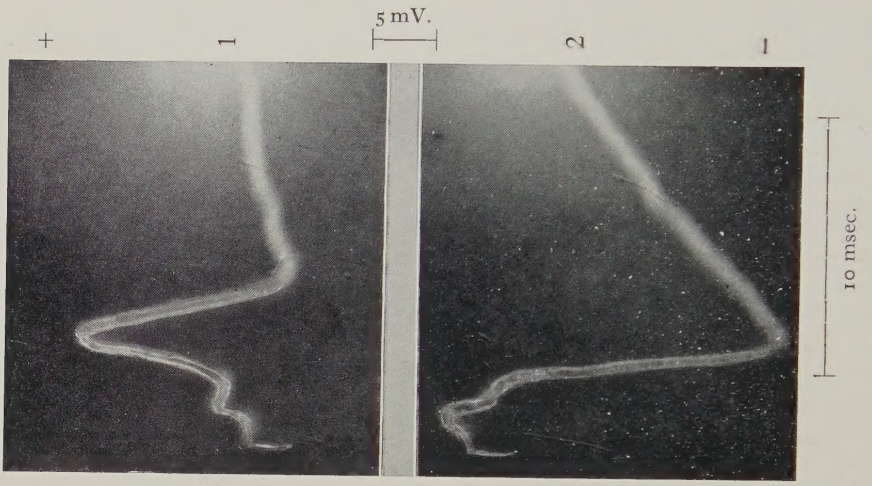
A transverse section of the mandible muscle is sketched on the left with the tendon dipping into solution. Part of the carapace has been cut away as illustrated by the dashed line *A*. *B* is the optional cotton connexion. Electrode 1, indifferent, is immersed in solution. Placement for negative area is designated by electrode 2. Electrode 3 shows normal placement on body of muscle. The action potentials obtained from the two placements are illustrated on the right. *A* 1–3 was recorded from muscle body with no connexion from carapace to solution. The deflexion is negative, about 20 mV. *B* 1–3, the same with cotton wick connecting shell to solution. Deflexion is positive, about 15 mV. *A* 1–2 was recorded from the negative area with no connexion. The deflexion is negative, about 40 mV. *B* 1–2 the same with cotton connexion from shell to solution. Deflexion is negative, about 15 mV.

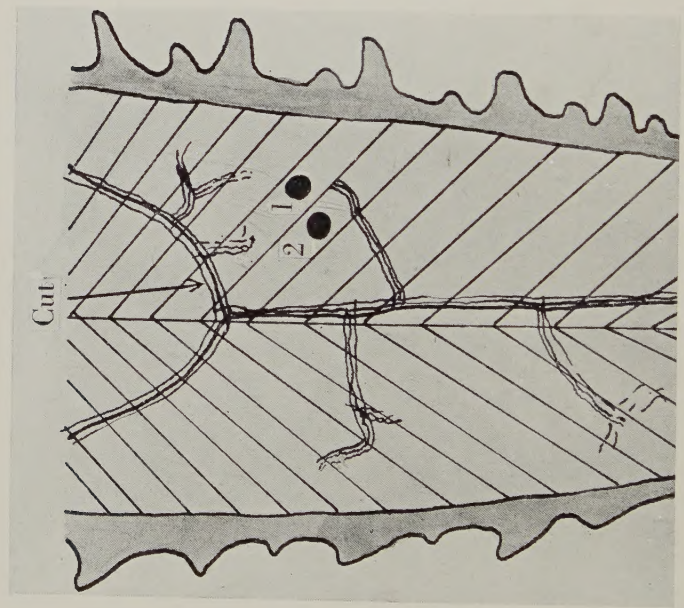
PLATE 8

The inner surface of the extensor muscle of the meropodite showing the nerve ramifications is illustrated on the left. After a nerve branch was cut, as designated in the figure, and probing electrode placed at 1 the upper spike was recorded. It is mostly positive. If the prober were placed at 2 the lower spike, negative, was obtained.



WIERSMA AND WRIGHT—ACTION POTENTIALS OF CRUSTACEAN MUSCLES





WIERSMA AND WRIGHT—ACTION POTENTIALS OF CRUSTACEAN MUSCLES

THE FORMATION AND STRUCTURE OF THE MICROPYLAR COMPLEX IN THE EGG-SHELL OF *RHODNIUS PROLIXUS* STÄHL. (HETEROPTERA REDUVIIDAE)

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(With Fifteen Text-figures)

INTRODUCTION

The micropyle and associated structures of insect eggs have received little detailed attention, although they are of great importance. For not only do they play a vital part in fertilization but also, since they penetrate the whole of the chorion layers, they form a most likely site of entry for ovicidal materials. They may also be of importance in the water-exchange and respiratory systems of the egg.

Beament (1946*b*) has already described in detail the properties, formation and permeability of the chorion of the heteropteron, *Rhodnius prolixus* Stähl. The unspecialized shell consists of seven layers, and the properties attributed to them enable such materials to be detected in more complex regions of the shell. It was, moreover, shown that in three specialized areas of the shell (the cap surface, the neck and the rear end) modifications were produced by variation in the relative thickness of the component layers and not by the secretion of new components.

It is the object of this paper to investigate the structure of the micropyle and the associated structures of the junction between the shell and its cap, in terms of the component layers of other regions of the shell, and by following the secretions of the follicle cells. In this way it is hoped to obtain a structural basis for the importance of such a complex in the physiological relationship of the egg.

THE LAYERS OF THE *RHODNIUS* EGG-SHELL

Beament (1946*b*) has shown that the unspecialized part of the shell of the *Rhodnius* egg is formed in the following way (Fig. 1).

The follicle cells in a deeply staining phase secrete the endochorion layers. These consist of:

(1) A series of tanned granules of protein, 2μ in diameter, with a high polyphenol content. This is the *inner polyphenol layer*.

(2) A layer of tanned protein, 1–2μ thick, and resistant to enzymes and to acids and bases, called the *resistant protein layer*.

(3) The *outer polyphenol layer*, which is similar to the inner layer but in which the granules are much smaller.

(4) The *amber layer*, which is the only coloured layer of the shell. It is formed by the addition of oil to tanned protein after the latter has been secreted. The oil is released from this product only after the action of the strongest hot acid.

(5) The *soft protein layer* forming the greater part of the thickness of the endochorion. Its material is similar to that of the resistant protein layer, but is more readily attacked by acids and bases. It contains diffusely scattered polyphenol elements. This layer will also be referred to as the *soft endochorion*, as opposed to the *resistant endochorion*, made up of the other four layers.

The exochorion is secreted after the follicle cells have undergone a transition to a non-staining phase. It consists of two layers of a lipoprotein ('chorionin') which is formed in the cells before secretion (cf. the amber addition product).

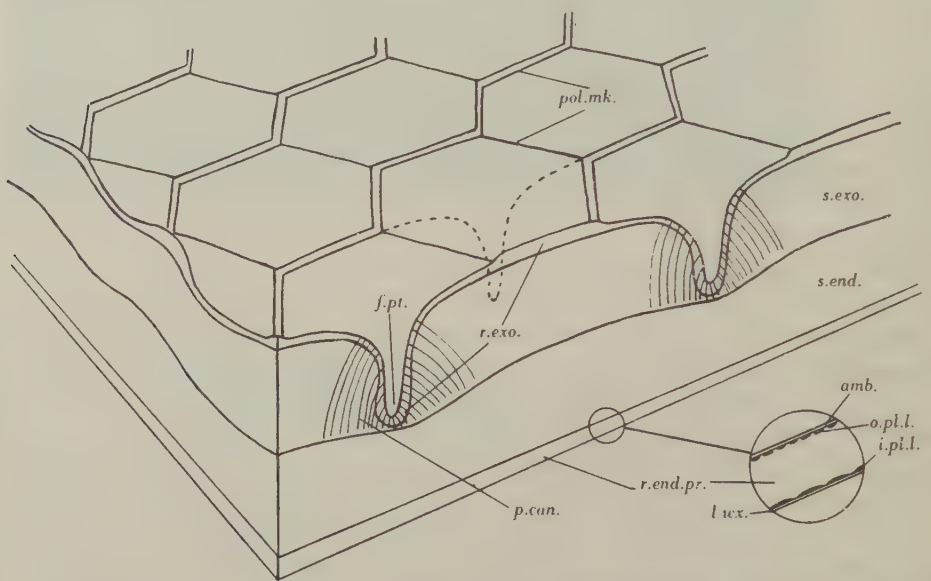


Fig. 1. Diagrammatic representation of a fragment of the *Rhodnius* egg-shell from the unspecialized region of the chorion, showing the polygonal markings of the surface (*pol.mk.*) and the follicular pits (*f.pt.*) at their centres. Inset: the layers of the resistant endochorion. *amb.* amber layer; *i.pl.l.* inner polyphenol layer; *o.pl.l.* outer polyphenol layer; *p.can.* pore canals; *r.end.pr.* resistant endochorion protein layer; *r.exo.* resistant exochorion layer; *s.end.* soft endochorion layer; *s.exo.* soft exochorion layer; *l.wx.* primary wax layer. (After Beament, 1946*b*.)

(6) The *soft exochorion* is a thick layer, which is mainly responsible for the sculptured appearance of the egg. It is impermeable to all but small ions and water, and is soluble in potash and in strong nitric acid, releasing the oily component of the lipoprotein.

(7) The *resistant exochorion* forms the outermost, thin layer of the chorion and is a more resistant form of chorionin.

Each binucleate follicle cell leaves a polygonal outline on the shell and there is a deep pit, the *follicular pit*, in the centre of each polygon, but confined to the exochorion layers. Pore canals are present in the exochorion layers only, running from the inside of the pits towards the endochorion; they do not penetrate to the

soft endochorion (Beament, 1946*b*) (Fig. 1). According to these characteristics the shell may be divided into a number of blocks of material, each the product of an individual follicular cell, and this method of analysis will be used in the following work.

The rims and seal complex

Fig. 2 shows a longitudinal section through the region where the rim of the cap is sealed on to the rim of the unspecialized shell. The main parts referred to below will be described briefly.

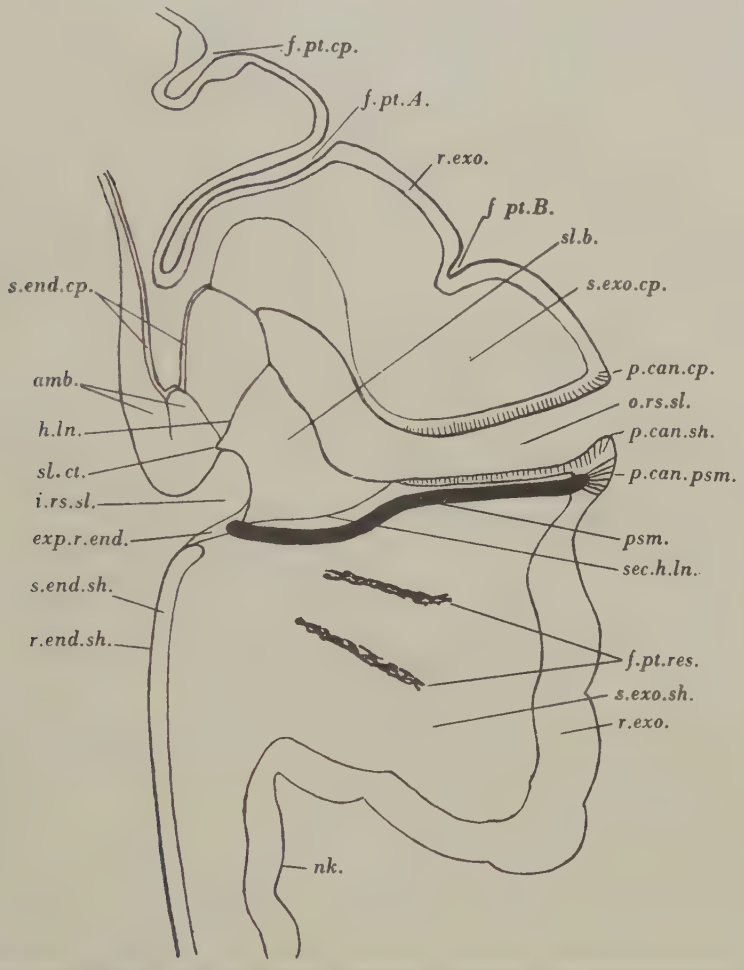


Fig. 2. Longitudinal section through the seal between the rims of the cap and main shell. *amb.* amber layer; *exp.r.end.* expanded resistant endochorion layer; *f.pt.A.*, *f.pt.B.* follicular pits of the *A* and *B* cells; *f.pt.cp.* follicular pit of the main cap surface; *f.pt. res.* residues of follicular pits in the rim of the shell; *h.ln.* hatching line; *i.rs.sl.* inner recess of the seal; *nk.* neck of the shell; *o.rs.sl.* outer recess of the seal; *p.can.cp.* pore canals of the rim of the cap; *p.can.psm.* pore canals of the pseudomicropyles; *p.can.sh.* pore canals of the rim of the shell; *psm.* pseudomicropyle; *r.end.sh.* resistant endochorion layers of the shell; *r.exo.* resistant exochorion layer; *sec.h.ln.* secondary hatching line; *s.end.cp.* soft endochorion layer of the cap; *s.end.sh.* soft endochorion layer of the shell; *s.exo.cp.* soft exochorion layer of the cap; *s.exo.sh.* soft exochorion layer of the shell; *sl.b.* sealing bar; *sl.ct.* sealing catch.

The circumference of the cap is expanded to form the rim of the cap. This expansion is produced mainly by an increased thickness of soft exochorion and secreted round a ring of very long, but otherwise normal follicular pits. On the inside of this rim the amber layer is folded inwards and thickened, but the soft endochorion protein layer and the resistant endochorion layers are extremely thin. The rim of the cap is therefore forked in section; the sealing bar which connects the rim of the cap to the shell is attached to the inside of this fork. It abuts on to the amber layer, and its junction with the cap is marked by the line of weakness, or hatching line along which the shell breaks at eclosion. The sealing bar is the thinnest part of the shell; it consists of a very thin inner resistant endochorion, together with a very thick amber layer—the exochorion layers are missing.

Below the sealing bar, the rim of the shell is again formed by an expansion of the soft exochorion, while all the inner layers are very thin. This rim is secreted around four rings of long follicular pits. The posterior three are filled in during secretion, but the anterior ring, set in the upper part of the rim of the shell, remain. At first, these appear (Fig. 11) to be normal follicular pits of exceptional length; there are some two hundred around the rim in *Rhodnius*. They are, apparently, present in most hemipteron eggs, and have been the subject of considerable controversy and misstatement.

Leuckart (1855) and others have stated that these pits are all micropyles, and the name 'samenbecher', or seminal cups, has been applied to them, especially in some other species of Heteroptera where they may be produced into processes rising from the rim. On the other hand, Gross (1901) and Heymons (1926) suggested that these were for aeration, though no other structures for the entry of spermatozoa were described. Johnson (1934), describing the rim of the egg-shell in *Notostira erratica*, depicted tubes at this level opening on to the inside of the shell by a funnel 5μ in diameter, but could not find any trace of their external opening and supposed that they were connected with the large cavities in the cap. He stated that twenty to forty may occur, scattered irregularly around the rim. Other distributions of 'micropyles' in hemipteron eggs are quoted by Heidemann (1911): eggs of *Brochymena* may have thirty to forty, *Euschistus* sixty or more, and *Belonochilus numenius* as few as five. Apparently the numbers are variable, and the distribution is irregular in all hemipteron eggs that have been described.

It will be shown below that the majority of the pits in the shell rim are not open to the external surface of the shell, neither do they penetrate to the surface of the egg cell. These cannot satisfy the function of micropylar apparatus, since they cannot permit the passage of spermatozoa through the shell for fertilization. (It is known (Beament, 1946*d*) that the *Rhodnius* egg is fertilized after the shell is complete, and, therefore, the existence of some form of micropyle is apparently imperative.) These closed tubes will be referred to as *pseudomicropyles*. However, a modification of about fifteen of these pits results in the formation of the *true micropyles* (Fig. 13). Each opens on the outer side of the egg-shell by a funnel-shaped orifice of about 2μ diameter and in the groove which occurs below the dorsal point of the rim. This groove can, therefore, be called the *spermatic groove*

(Figs. 11, 13). On the inner surface, the micropyles penetrate the resistant endochorion, opening by a small aperture with a diameter of less than 1 μ (Fig. 12). The inner openings are on the same level as the blind ends of the pseudomicropyles, but the remainder of the tubes lie in a plane approximately 5 μ posterior to them.

METHODS

(1) *Cutting sections*

Most completed egg-shells, including that of *Rhodnius*, are too brittle to give serial sections by standard methods of procedure. Hence, for most of this study the method of observing the cut edge of shells cut longitudinally while frozen has been used (Beament, 1946*b*). However, for the elucidation of the formation and structure of the rims, seal and micropylar structures, it was essential to obtain a suitable technique. All stages of oocyte development and shell formation up to the time when the exochorion layers are being secreted can be cut by normal methods (e.g. wax and celloidin embedding after fixation). On the other hand, the exochorion material hardens soon after secretion, especially with dehydration, and the shell then fragments when sectioned.

It has been shown (Beament, 1946*b*) that although potash solutions attack and dissolve the exochorion material, previous immersion in picric acid solution will prevent this. However, though solution does not take place, the shell substance is considerably softened by such action and remains so after drying. For cutting sections of eggs with any exochorion, ovarioles were fixed in aqueous Bouin's solution for 3 days and then dissected in the fixative. Incomplete oocytes were returned to the fixative, and those which had apparently reached full size were punctured in the side with a fine needle to ensure the complete fixation of the egg contents. After a further 3 days the complete oocytes were treated as follows:

The rear end of each egg was cut off about 0·5 mm. from the end, and mounted in glycerine, whence the degree of formation of the exochorion could be distinguished. Eggs without exochorions, or with less than about 2 μ of exochorion, were embedded and sectioned in the normal way, while those with thicker chorions were immersed in cold normal potash for about 10 min. and then washed in water and embedded as before. The picric acid in the Bouin's solution apparently 'fixed' all the proteins in the follicle cells as well as in the shell layers so that these sections appeared to be quite normal. They were, however, compared with similar (though fragmented) sections which had not been treated with potash, in order to confirm the histology of the follicle cells, and to ensure that the morphology of the minute structures in the rims was unaltered.

(2) *Staining in inorganic solvents*

Much of the information on the structure of the seal has been obtained by observing the action of solvents on the various components of this region and comparing their properties with those already determined for components of the unspecialized part of the shell. For this purpose it was desirable to have some part

of the material under observation coloured by staining, and to use this as a reference point. All usual stains are broken down by such solvents as concentrated potash and strong nitric acid, while the only stain which remains coloured in most solvents (picric acid) has already been shown to alter the properties of the layers considerably. For this particular purpose it was found that if half-shells were soaked in a Universal Indicator (B.D.H.) for 2 hr., and then dried in the air before placing in the solvent, some protein layers retained a marked colour in any of the solvents used, and this considerably facilitated the elucidation of the complex.

THE FORMATION AND DETAILED STRUCTURE OF THE SEAL

The shell can be considered as a series of units, each the product of a single follicle cell; the formation of the rims and seal will now be followed in terms of the activity of each specialized follicle cell in this region. It will be found that, in the completed shell, the units are more distinctly marked in this part of the shell than in any other.

Figs. 3–5 represent longitudinal sections through the cells of the follicle over the region where the seal is to be formed. These cells will be referred to by a series of letters (passing from the cap to the shell) as follows:

Z: the cells of the central cap region.
A, *B*, *C*, *D*, *E*, *F*, *H*, *K*: the specialized cells of the seal.
M_1, M_2, M_3, M_4: the cells of the shell rim.
N_1, N_2, N_3: the cells of the neck.

Since the shell is radially symmetrical it must be remembered that each of these cells is representative of an annulus of similar cells around the oocyte at that particular level, and that secretions will take the form of annuli, produced, for example, by the *ring* of *B* cells.

Before secretion, the cells *A*–*K* do not differ in histology or morphology; they are apparently differentiated from the unspecialized cells during a series of movements associated with the production of a slight fold in the vitelline membrane at the site of the seal (Figs. 4, 5). These movements take place principally around the rings of cells *A*, and *K* and M_1, which are displaced inwards towards the yolk, while the central cells (*E*) are raised slightly (Fig. 5). The movement of the *K* cells relative to their original position is of the order of 20μ along the radius of the annulus, so that this ring of cells is compacted (see p. 228). At the same time, the cells *B*, *C* and *D*, and *F* and *H* lose all but fragmentary contact with the surface of the oocyte (Fig. 5).

It is during these movements that shell secretion starts, and it appears that this activity takes place only in those cells which are in contact with the surface of the oocyte. Cells *B*, *C*, *D*, *F* and *H* are therefore dormant at this stage, but as the shell is secreted, the increase in surface area allows them to resume contact with their substrate and to secrete (see below).

Together with all the other cells of the follicle, the *A*, *E*, *K* and *M* cells secrete the inner polyphenol layer and the protein of the resistant endochorion layer

(Fig. 5). This is followed by the outer polyphenol layer in the case of all cells but the *K* series. The *K* ring of cells will eventually form the pseudomicropyles. The process of pit formation (with the exception of pore canals) by the *K* cells is similar to follicular pit formation in the unspecialized shell, but it starts in the depth of the resistant protein layer which is slightly thickened opposite the cells *K* and *M* (Figs. 5, 6). The blind ends of the pits formed by the *K* cells are separated from the surface of the oocyte by a distance of about 0·5 μ, and at this point the substance of the resistant endochorion protein layer is permeated by minute polyphenol granules, contrasting strongly with the composition of this layer in all other parts of the shell. On the other hand, over the areas secreted by the *E* and *A* cells, the resistant endochorion is very thin and the two layers of polyphenol granules cannot be readily resolved.

In following the subsequent secretions of these specialized follicle cells, it can be seen that their displacement from 'normal' positions corresponds to gross disturbance of their activity, compared with the unspecialized cells. All other materials which form separate layers in the unspecialized shell, are secreted as such. However, in some places they are compounded to form new substances, and polyphenols are often produced at an unusual stage (Table I).

The *A* and *E* cells now secrete protein material similar to the cap cells, while the *K* cells produce soft lipoprotein identical with the material of the soft exochorion (Figs. 6, 10). The *M* cells, together with those of the neck, form a submicroscopic layer of amber material, and then lay down protein of the soft endochorion type; these cells have identical staining reactions with the generalized cells of the shell at this time. Very shortly after the production of the innermost secretions of the *A* and *E* cells, the *B* cells start to secrete. Their product is also a protein material, but it differs from the substance secreted by the *A* and *K* cells, for it has a slightly greater affinity for water-soluble stains (probably indicating a less degree of tanning and associated processes). Small quantities of unfixed polyphenol material are also present (these *B* cells did not contribute polyphenol to the resistant endochorion).

While the *A*, *B* and *E* cells are secreting protein, they accumulate numerous oily droplets in the region farther from the shell, very similar to the oily droplets found in the cap cells (Beament, 1946*b*). Towards the end of this secretory phase (Fig. 6), the oil disappears and the layer of protein already secreted is transformed to amber material. It is interesting to note that the material secreted by the *E* cells (the sealing bar) constitutes the thinnest region of the whole shell. This is perhaps counterbalanced by its composition, for it consists almost entirely of the extremely resistant amber material which is thicker here than anywhere else in the shell. The only exception to lipidization in the sealing bar is the resistant endochorion.

The region formed by the *B* cells up to this stage is less lipidized, and more soluble in chlorated nitric acid, than the surrounding amber material. The rate of secretion of these cells is not uniform, for while the cap cells have produced a membrane about 2 μ thick, the *A* cells have secreted about 4 μ, and the *E* cells approximately 7 μ of amber material. However, this extremely rapid rate of secretion

on the part of the *E* cells completes their active life, for they cease secretion after the sealing bar is formed.

The predetermined line of weakness, or hatching line, is already present and is marked by the site of junction first of the cells *A* and *E* and later *B* and *E*. It is,

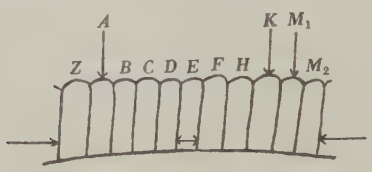


Fig. 3

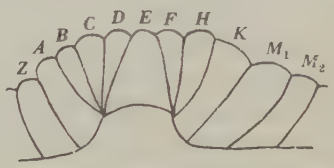


Fig. 4

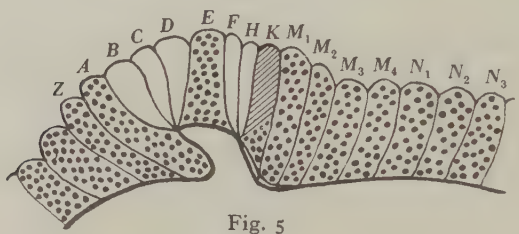


Fig. 5

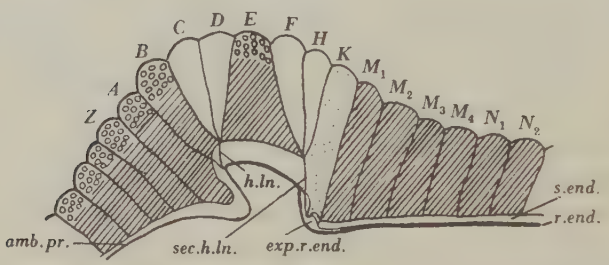
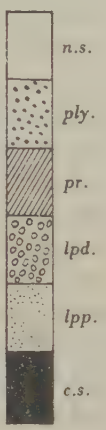


Fig. 6



Key to Figs. 3–8

Key to Figs. 3–8. *n.s.* cells not secreting; *ply.* cells secreting polyphenols; *pr.* cells secreting protein; *lpd.* cells accumulating oil and lipidizing the protein to amber material; *lpp.* cells secreting lipoprotein; *c.s.* cells which have ceased secreting and are dying.

Fig. 3. Diagrammatic representation of a longitudinal section through the follicular cells at the site of the seal and rims complex (each cell represents an annulus around the oocyte long axis). *Z*, cell forming the cap; *A*-*K*, cells forming the rims and seal; *M*, cells of the rim of the shell; the arrows indicate the determination movements.

Fig. 4. Diagrammatic representation of follicle cells of the seal during determination movements. *Note.* Cells *B*, *C*, *D* and *F*, *H* have moved out of contact with the oocyte surface. The secretion of the chorion starts at approximately this point.

Fig. 5. Secretion of the seal region. Determination movements are now complete and the cells have secreted the initial layers of the shell. Cells *Z*, *A*, *E*, M_{1-4} and N_{1-3} (of the neck) secreting the outer polyphenol layer, cells *B*, *C*, *D* and *F*, *H* not active, and the *K* cell (pseudomicropyle cell) is secreting the expanded granular resistant layer.

Fig. 6. Secretion of the seal region. The *B* cell is now active, and together with the *A* cell is secreting protein (*amb.pr.*) to be lipidized to amber material. The *E* cell has already formed about half the thickness of the sealing bar, while the *M* and *N* cells are completing the reduced soft endochorion (*s.end.*). The *K* cell has completed the expanded resistant endochorion leaving the inner end of the pseudomicropyle and is secreting lipoprotein. The paths of retreat of the inactive cells (*C*, *D*, *F*, *H*) are marked by the primary (*h.ln.*) and secondary (*sec.h.ln.*) hatching lines.

therefore, the path of retreat of the tips of the non-functional cells *B*, *C* and *D* and later *C* and *D* (Figs. 5, 6), whose presence appears to weaken the co-ordination between the secretions of the two active cells. Because the *B* cells become active shortly after the cells *A* and *E*, there is a small projecting wedge on the sealing bar which fits closely into a groove on the boundary of the material secreted by the *A*

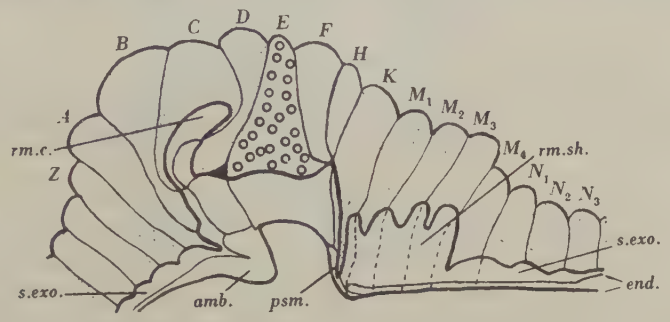


Fig. 7. Secretion of the seal region. The endochorion layers (*end.*) are now complete, and almost all the cells are secreting the soft exochorion (*s.exo.*). Note the inner end of the long follicular pit of the *A* cell. The *C* and *D* cells have now started to secrete and are forming the overhanging rim of the cap (*rm.c.*). The *E* cell is completing the lipidization of the sealing bar, while the *M* cells are forming the rim of the shell with its long follicular pits (*rm.sh.*). The *K* cell now has a very long villus in the cavity of the pseudomicropyle (*psm.*). The *F* and *H* cells are not yet active. *amb.* the amber layer.

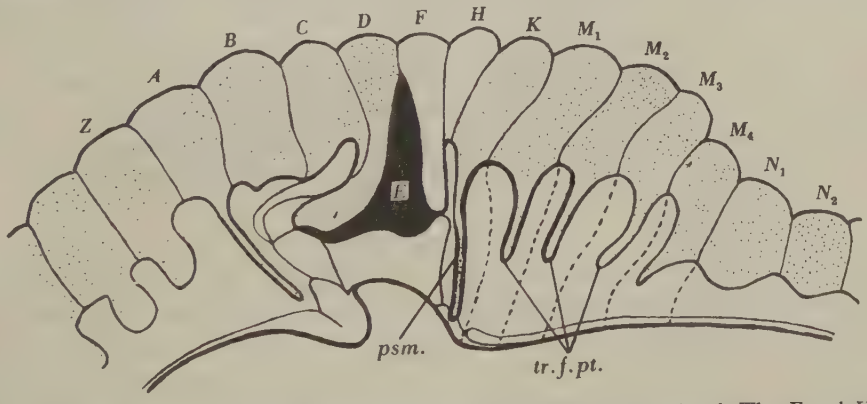


Fig. 8. Secretion of the seal region. The exochorion layers are half completed. The *F* and *H* cells are the only ones not secreting, while the *E* cells, cut off by the outer ends of the *D* and *F* cells, are now dying. The rims are assuming their final form. *psm.* pseudomicropyle; *tr.f.pt.* transitory follicular pits.

cells. This is important in the mechanism of hatching (Fig. 10). It is also noteworthy that a secondary predetermined line of weakness is produced along the line of retreat of the other set of non-functional cells *F* and *H* (Fig. 6). Occasionally, after the front ends of incomplete shells had been boiled in chloroform, pressure on the cap from within resulted in splitting along this secondary line of weakness instead of the normal one. However, such a phenomenon was not found in any of the naturally vacated shells.

With the completion of the amber layer, the cells undergo a transition phase during which the cells *A* and *B* produce a minute amount of soft protein material similar to that present in the cap (Fig. 2 and see Beament, 1946*b*). It is again, perhaps, a vestigial form of the processes involved in the production of the unspecialized shell. The cells *A*, *B* and *C* and those of the rim and neck, *M* and *N*, assume the characteristics of chorionin secretion (p. 214) and now produce soft lipoprotein (Fig. 7). The *C* cells, using the thin layer of soft protein as a substrate, produce a long overhanging lip. At the same time, the *D* cells, starting on the amber material secreted by the *B* cells, produce a trace of soft protein, and then also secrete soft lipoprotein which fills the recess of the overhanging lip formed by the *C* cells (Figs. 7, 8).

During this time, the *K* cells continue to secrete soft exochorion material, leaving their long cytoplasmic processes which penetrate into the resistant layer of the endochorion. Similarly, the rim cells *M* have left long processes in the soft lipoprotein (Fig. 8). It is about half-way through their phase of soft lipoprotein production that the unspecialized cells over the main shell change to the secretion of the exochorion (Beament, 1946*b*). The rates of secretion in all parts of the shell are apparently so co-ordinated that, although at any time during the secretion of the inner layers, different rings of cells may be secreting different materials, all regions complete their soft exochorion layer and arrive at the anteterminal phase at the same time. Immediately before this, the *M* cells in the rim withdraw their villi and fill the cavities of their follicular pits with an extremely soft vacuolated lipoprotein material which has greater solubility in aqueous potash than any other part of the shell. The sites of these pits are marked in the completed shell by their large content of chloride (Fig. 2); they are the only parts of the shell to stain jet black after treatment with 5% silver nitrate solution in the presence of nitric acid, followed by exposure to light. This would indicate that cellular debris is left when the villi are withdrawn.

In the final stage (Fig. 9) the resistant exochorion is secreted over the whole shell with the exception of the outer surface of the sealing bar and a small region of the initial amber material secreted by the *B* cells. The lack of resistant layer here is due to the fact that this surface is covered by the necrotic remains of the *E* cells (Fig. 8), and therefore no secretion takes place. It is during this final phase that the long cytoplasmic villi are withdrawn from the pseudomicropyles, and the outer ends sealed, with the exception of a small bunch of pore canals (Figs. 2, 11) which give communication with the surface of the shell. The pseudomicropyles are not lined with resistant lipoprotein like the other follicular pits of the shell and cap, since the villi are withdrawn before the terminal phase of secretion. When placed in potash they dissolve away completely, instead of leaving the normal thin layer of resistant material. Only the outermost part of the cavity lies in the resistant layer formed by the *K* cells, and after immersion in potash solution, this remains as a lamina containing small cavities at the sites of the pseudomicropyles.

It is during this final phase that the two remaining groups of cells (*F* and *H*) pour out their secretions (Fig. 9). They produce resistant lipoprotein over the soft

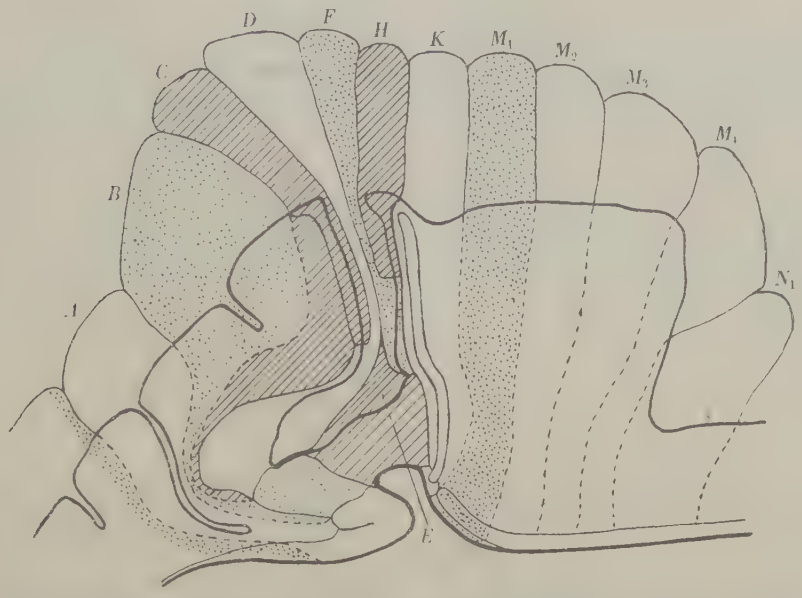


Fig. 9. Secretion of the seal region, showing the areas of the complex which are secreted by the various annuli of cells. This should be compared with Fig. 2 where the actual delineation of areas is shown. *Note*. While the cells in this figure represent the final appearance of the follicle cells, the shading of areas merely represents a cell and its respective secretion. It has *no* relation to the key used for Figs. 3–8.

Table 1

Cell	Secretions
Z	*a*...*b*...*a*...*b*.....*o*.....*b*...*c*.....*d*
A	*a*...*b*...*a*...*b*.....*o*.....*b*...*c*.....*d*
B	*b*...*b*...*o*...*c*.....*d*
C	*c*.....*d* and *a*
D	*b*...*c*.....*d* and *a*
E	*a*...*b*...*a*...*b*.....*o*
F	...*d* and *a*
H	...*d* and *a*
K	*a*...*b*.....*c*.....*d*
M	*a*...*b*...*a*...*b*...*b*...*c*.....*o*.....*d*
N	*a*...*b*...*a*...*b*...*b*...*c*.....*o*.....*d*
S	*a*...*b*...*a*...*b*...*b*.....*o*.....*c*...*d*

a, polyphenols; *b*, protein; *c*, soft lipoprotein; *d*, resistant lipoprotein; *o*, lipidization.

The secretions of cells in the specialized areas of the shell (and see Beament, 1946*b*), compared with the cycle of secretions from the unspecialized cells. The arrows indicate the points at which a cell starts to secrete, using the secretion of another cell as its substrate. *Z*, cap cells; *A–H*, specialized cells of the seal; *K*, pseudomicropyle cells; *M*, cells of the rim of the shell; *N*, cells of the neck; *S*, cells of the unspecialized shell.

lipoprotein units containing the pseudomicropyles, and so give a resistant layer to the upper surface of the rim of the shell. The resistant layer which they secrete, and that formed by the *C* cells, is extremely porous and heavily impregnated with polyphenols (Fig. 2), so that when the completed egg is immersed in ammoniacal silver nitrate, these are the only areas of the *surface* which stain with the typical pink-brown coloration (see micropyles, p. 226). The pore canals formed by the *F* and *H* cells are, of course, confined to the layer which they secrete, and do not penetrate to the lumen of the pseudomicropyles; the canals are of the larger variety found in thickened resistant exochorion in other parts of the shell (Beament, 1946*b*).

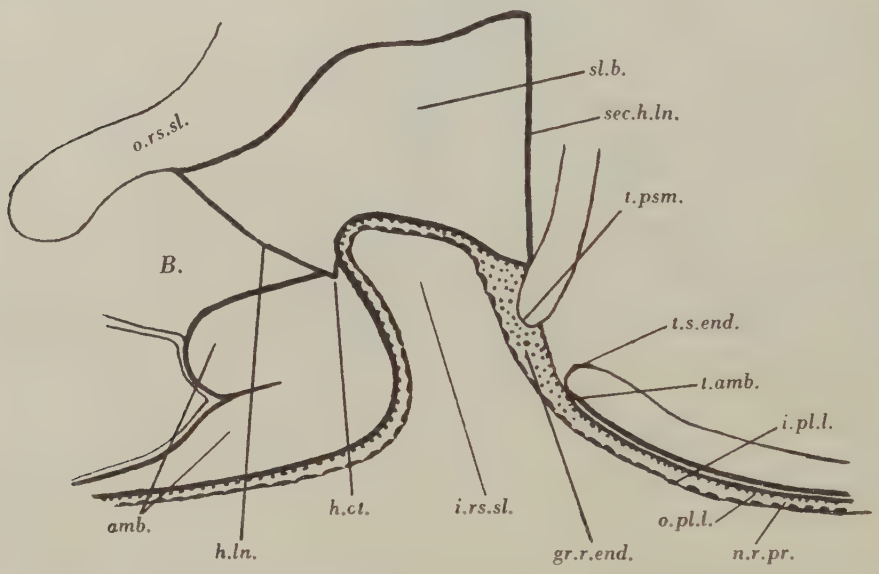


Fig. 10. Detail of the inner end of a pseudomicropyle in longitudinal section. The amber material is outlined heavily. *amb.* amber layer of the cap; *B*, the semi-amber specialized material formed by the *B* cells; *gr.r.end.* expanded granular resistant endochorion; *h.ct.* hatching catch; *h.ln.* primary hatching line; *i.pl.l.* inner polyphenol layer; *i.rs.sl.* inner recess of the seal; *n.r.pr.* normal resistant protein layer; *o.pl.l.* outer polyphenol layer; *o.rs.sl.* outer recess of the seal; *sec.h.ln.* secondary hatching line; *sl.b.* sealing bar; *t.amb.* termination of the amber layer in the shell; *t.psm.* end of pseudomicropyle; *t.s.end.* end of the soft endochorion protein layer in the shell.

Variations in the pseudomicropyles

Not all the pseudomicropylar pits are open to within about 1 μ of the surface of the shell. In a few cases these pits end at points opposite the junction between the regions secreted by the *F* and *H* cells. The remainder of the path which would have been described by the normal follicular pit is filled in with soft lipoprotein material containing polyphenols, and it is by staining in this way with ammoniacal silver nitrate that the subsequent path of the follicular villi can be traced (Fig. 11). The filling of these pits is similar to the closure of the pits secreted by the *M* cells of the rim of the shell.

The function of the pseudomicropyles

No obvious role can be allotted to the pseudomicropyles in connexion with fertilization; it is, however, important to consider them further, since they may possibly convey liquids and gases into close proximity with the embryo (Fig. 10).

EXPERIMENTAL

Whole waterproof eggs (Beament, 1946*a*, 1946*c*), removed from the ovaries and dried for several hours, were immersed in aqueous stains for 24 hr., and the rims were then dissected from the eggs and observed in liquid paraffin (to prevent the diffusion of any aqueous stain in the shell cavities). The pseudomicropyles were unstained. Other eggs of the same stage were immersed in a solution of Sudan III

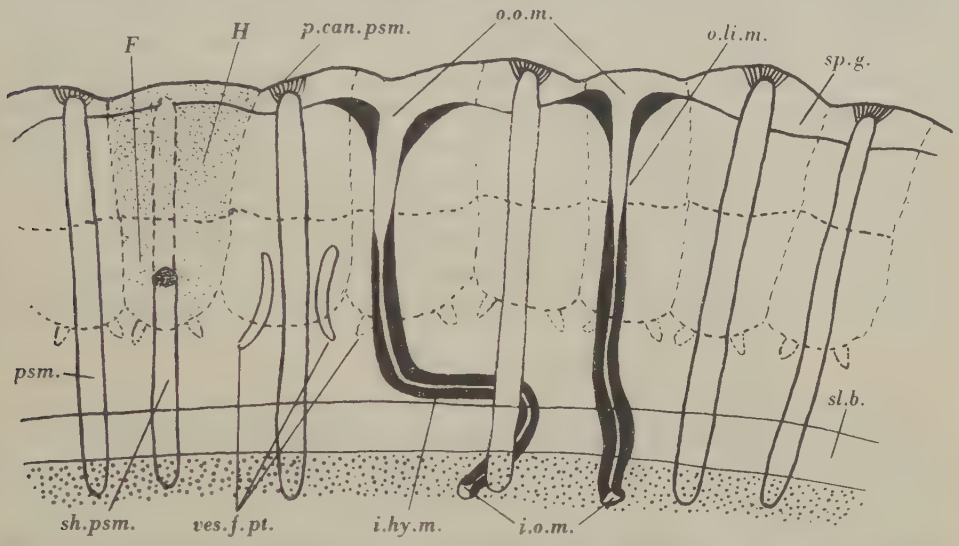


Fig. 11. Plan of arc of the rim of the shell, seen from above, showing the path of two micropyles. *F* and *H*, very porous lipoprotein secreted by the *F* and *H* cells respectively; *i.hy.m.* inner part of micropyle lined with hydrophylic material; *i.o.m.* inner opening of the micropyle; *o.li.m.* outer part of micropyle lined with lipophylic material; *o.o.m.* outer opening of micropyle; *p.can.psm.* pore canals leading to pseudomicropyle; *psm.* normal pseudomicropyle; *sh.psm.* short variety of pseudomicropyle; *sl.b.* sealing bar; *sp.g.* spermatic groove; *ves.f.pt.* 'vestigial' follicular pits, possibly formed by the *F* and *H* cells.

in light petroleum, and observation of their rims (mounted in glycerol) showed that the lumen of the pseudomicropyles was filled with stain after 24 hr. immersion. Similar results were obtained with suitably coloured benzene and xylene. It therefore appears that oily liquids can penetrate the shell and fill the pseudomicropyles, and it was important to discover whether the oil was penetrating through the lipophilic chorionin, or along the small group of pore canals which make connexion with the shell surface.

In order to clarify this point, eggs were immersed in stain in an evacuated vessel, by using the apparatus described by Beament (1946*b*, 1946*c*). In this way air

spaces in the egg were emptied, and the readmittance of air into the vessel might force liquid through the pores into the pseudomicropyles. Under these conditions the cavities of the pseudomicropyles were filled with both aqueous and oily stains almost immediately.

CONCLUSION

It appears from these experiments that, after waterproofed eggs have been removed from the ovaries and desiccated sufficiently to remove water from the shell, there is free passage for air from the surface of the shell along the pore canals. These pore canals are, presumably, lined with chorionin and will therefore be tiny hydrofuge capillaries, so that aqueous material will not pass along them at all easily. On the other hand, their surface will be wet readily by oily material, and this will traverse the pores, providing the air in the pseudomicropylar cavity can be displaced. When eggs are merely immersed in oils, the air must either be dissolved in the oil or taken into the egg cavity, possibly by respiration; it is a slow process, however, and, from the above experiments, one which does not take place in water. Since air is more soluble in oil than in water, the air in the lumen may be dissolved by the oil as it enters, but neither respiration nor solution seems to produce sufficient drop in pressure to suck water along the lipophilic capillaries, and the entry of oil must depend primarily on its ability to wet the surfaces of the pore canals.

It is, however, apparent that the pseudomicropyles could, and probably do, act as respiratory structures and are the points in the shell where air can penetrate most readily, but it is doubtful if they could supply the whole oxygen needs of the egg.

The true micropyles

It has been stated above (p. 217) that the true micropyles lie slightly posterior to the plane of the pseudomicropyles for most of their length (Fig. 13). Each is the product of a single follicle cell, and the micropylar tube appears to be a modified follicular pit corresponding to a specialized cell at that particular site in the follicle. It may be noted at this stage that the position of these micropyle-forming cells does not correspond to either the K or M_1 ring. The cells would, therefore, appear to be isolated units occupying an intermediate position as opposed to being units of a uniform annulus of cells. The activity of these cells during secretion differs from the normal K cells in the following details:

A 'follicular pit' is present from the initial stage in shell secretion, so that the innermost polyphenol and resistant protein layers are perforated by a funnel-shaped opening about 1μ in diameter. The granular resistant endochorion is formed as in the case of the pseudomicropyles, and the cells then produce soft lipoprotein in every way similar to the normal K cells, so that the lumen of the micropyle at this stage is similar in size to that of the pseudomicropyles. However, as the long follicular villus is withdrawn, it lines the innermost half of the tube with a proteinaceous material containing polyphenol granules. This material stains moderately with ammoniacal silver nitrate solutions, and slightly with water-soluble stains and protein indicators. The lumen of the inner portion of the micropyle is thus reduced to a tube of approximately $0{\cdot}5\mu$ in diameter (Figs. 11–13). The outer region of the tube

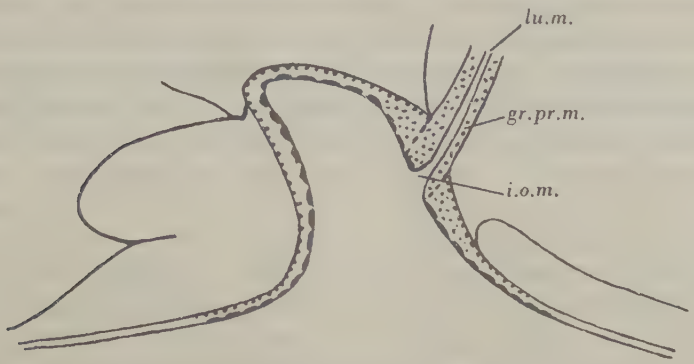


Fig. 12. Longitudinal section through the inner portion of the seal region, passing through a true micropyle. *gr.pr.m.* granular protein lining of the micropyle; *i.o.m.* inner opening of the micropyle; *lu.m.* lumen of the micropyle.

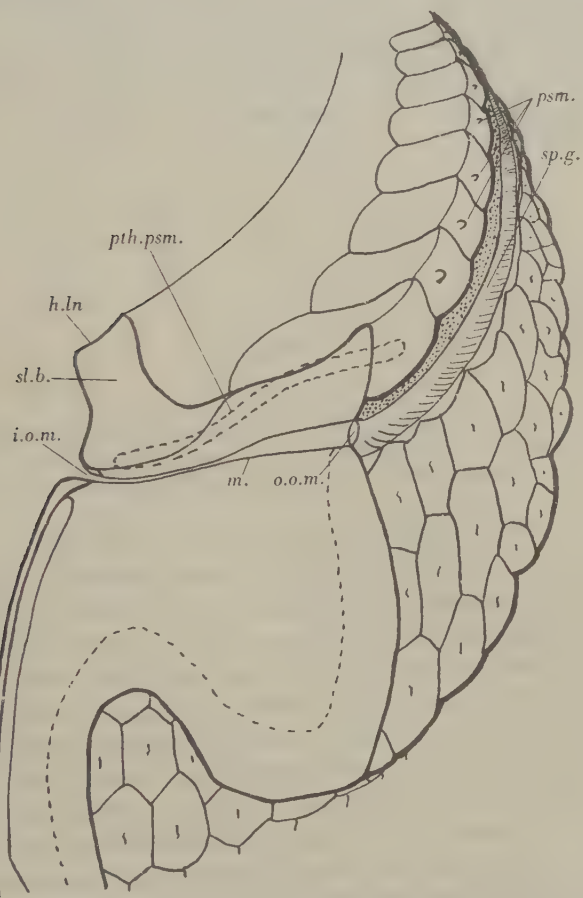


Fig. 13. The rim of the shell, with the cut edge passing through a true micropyle. *h.ln.* hatching line; *i.o.m.* inner opening of the micropyle; *m.* lumen of the micropyle; *o.o.m.* outer opening of the micropyle; *psm.* outer ends of the pseudomicropyles; *pth.psm.* path of a pseudomicropyle; *sl.b.* sealing bar; *sp.g.* spermatic groove.

is lined with resistant exochorion lipoprotein, secreted at the same time as this product over the rest of the shell, but the formation of this compound does not result in the constriction of the outer half of the tube. The completed pit is not sealed, but opens on to the spermatic groove by a funnel-shaped orifice at a level approximately 5 μ below the ring of pseudomicropyles. These tubes are the only structures which allow free access between the surface of the oocyte and the external environment.

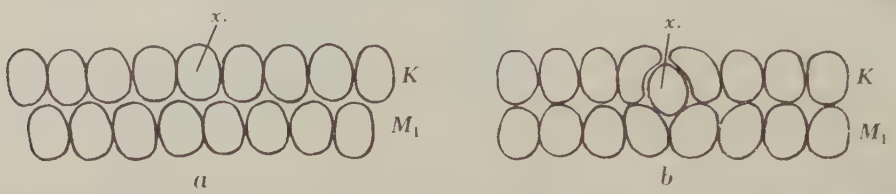


Fig. 14. Diagrammatic impression of the movements in the follicle cells around the rims, which may determine the micropyle forming cells. *a*, the *K* (pseudomicropyles forming cells) and M_1 (rim forming cells), in two rows around the oocyte, before determination movements or secretion; *b*, the compression on the *K* ring during the folding movements have displaced one cell (*x*.) into an intermediate position between the *K* and M_1 cells; this is the micropyle-forming cell.

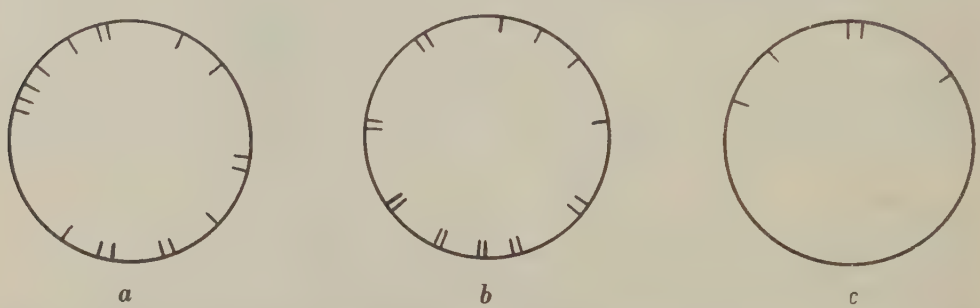


Fig. 15. The distribution of the micropyles around the rim of the shell, showing the irregular pattern adopted. *a* and *b*, normal distribution of micropyles; *c*, a typical distribution of micropyles in sterile egg laid by an aged female, showing the small number of micropyles found only in one side of the rim.

The determination of mycropyle-forming cells

During the formation of the initial fold in the oocyte membrane (p. 218) and the secretion of the inner polyphenol layer, the *K* ring of cells is apparently suppressed radially through a relative distance of about 20 μ. There are approximately 200 cells in this ring which describes a circle of about 250 μ in radius. Supposing no shrinkage takes place in the *K* cells, there will be room for 20–250 fewer cells in the smaller ring. In other words, approximately sixteen cells will be displaced from the *K* ring. If this displacement took place towards the posterior end of the shell, the displaced cells would be in the positions which are occupied by the micropyles. A diagrammatic impression of such movements is given in Fig. 14. Micropyle-forming cells, of course, might be so derived from the slight compression of the M_1 ring, or they may be present in the micropylar position from the time when the follicle cells are first arranged in a single layer around the oocyte, but evidence can be advanced for the above theory of determination as follows:

(1) The shell units containing the micropyles are morphologically more akin to the pseudomicropylar units than they are to any other segments of the shell.

(2) The inner ends of the micropyles open on to the shell surface at almost the same level as the inner ends of the pseudomicropyles, suggesting that the micropyle cells are only slightly displaced from the plane of the *K* ring at this stage in shell secretion.

(3) The micropylar tubes, in contrast to the pseudomicropyles, do not follow a straight path through the shell. The tube is straight in its outer portion, but where it passes into the endochorion, its path may be quite irregular. It may thus show all variations between a slightly undulating course, and a path which bends sharply in the endochorion, passing beneath two pseudomicropyles, and often returning before resuming a course radial to the shell. Such a path would be readily explained if the micropylar cells were being squeezed into its final position during the secretion of this part of the tube.

Table 2. *The number of micropyles in eggs of various aged females*

First egg batch	After 4 weeks egg-laying	Sterile eggs from old females
12	6	1
14	9	2
14	12	3
15	12	3
15	13	4
15	14	5
16	16	5
16	16	7
16	17	Av. 3·8
16	19	
17	Av. 13·4	
17		
19		
20		
Av. 15·8		

(4) The number of micropyles is not constant: it varies between six and twenty in the shells examined, and Table 2 shows that the average number per shell is fifteen—a remarkably good confirmation of the above calculation. The proposed mechanism for the determination of micropyles explains this variation in numbers, which would depend on the amount of shrinkage in the *K* ring. It also provides a reason for the irregular distribution of these tubes; there would be no reason for cells to be displaced at regular intervals (see Fig. 15), or determined by some inherent plan of radial or bilateral symmetry (which appears to be the manner in which most biological structures are determined).

(5) The eggs laid by young females contain, on the average, more micropyles than those produced by females after several weeks of egg production (Table 2). If the displacement of the micropylar cells depends on the general activity of the follicle cells, then the cells of older females must be less 'virile'. It has been found (Beament, 1946*d*) that about $\frac{1}{4}$% of the eggs from the cultures were sterile, though

produced by fertilized females. It was found that these were the product of aged females, and an examination of the rims showed that the average number of micropyles present in these sterile eggs was four (Table 2 and Fig. 15*c*), though the pseudomicropyles and all other parts of the shell appeared to be normal. Where greater numbers of micropyles were present they appeared to be crowded into half or two-thirds of the circumference of the rim. These sterile eggs show an extreme case of the decrease in micropylar numbers with the ageing of the female. During the passage of these eggs through the lower female genital ducts, one side of the spermatic groove will be presented to the opening of the spermathecae, and will receive a small quantity of spermatozoa to fertilize the egg. Where there are few micropyles, or where they are crowded into one side of the rim, the sperm may not be able to migrate round the groove sufficiently to find and enter a micropyle. It is therefore possible that these eggs from old females may be sterile on account of the lack of micropyles.

DISCUSSION

A consideration of the materials secreted by the specialized follicle cells of the seal region (and see Table 1 and Fig. 9; compare Figs. 2 and 10) shows that all the cells secrete modifications of a proteinaceous substance, and that the formation of the complex does not involve the production of any compound other than those secreted by the cells forming the unspecialized portions of the shell (Beament, 1946*b*). Moreover, the order of secretion appears to be almost the same as that for the main shell and cap, though phases of the secretory cycle may be omitted, and with the exception that polyphenols may appear at any time in the cycle of secretion of a specialized cell. Where the specialized cells do not start to secrete until after adjacent cells have produced some portion of the shell, there is some correlation between the material with which they commence secretion, and the substrate on which they are placed at that time.

Thus, while the secretory cycle of the *A* cells follows the normal pattern, the *B* cells do not produce polyphenol or resistant protein layers; the substrate on which they start secretion is protein (subsequently lipidized by the *A* and *E* cells), and they start secretion with protein which is lipidized, and the normal cycle is then completed. The substrate on to which the *C* cells start secretion is the very thin layer of soft endochorion protein by the *B* cells. Their initial product is soft lipoprotein—the next phase in the normal cycle. The *C* cells produce polyphenol in the pore canals of their last product, the resistant exochorion; this is an example of a product being produced out of the normal position. The *D* cells start secretion on a substrate of amber material (*B*) and produce first protein and then the normal exochorion layers.

The *E* cells are normal in their activity up to the lipidization of their amber layer. But with the completion of the sealing bar, secretion ceases, possibly due to the fact that they lose contact with the tunica propria covering the ovariole, and are cut off by the outer ends of the *D* and *F* cells. The secretions of the *F* and *H* cells consist solely of resistant lipoprotein containing polyphenol material, and the

substrate for this secretion is soft lipoprotein, so that they form merely the last phase of the sequence.

The chain of secretions from the *K* cells represents a special point of interest. The initial polyphenol and protein layers are normal, but the subsequent phases are lacking and the cells pass straight to the secretion of soft lipoprotein. In doing so, the substrate on to which the soft lipoprotein is deposited is a protein layer as in all other regions of the shell, and in omitting three of the secretory phases, the attachment of lipoprotein to protein has been retained. The remainder of the sequence is normal.

Apart from the apparent correlation between the phase of secretion and the substrate at that time, the rigid cycle of secretions may be adhered to for purposes of obtaining a complete adhesion between the respective layers of the shell, and so the shell retains strength with a degree of flexibility.

The formation of any form of follicular pit appears to be correlated with the existence of an initial depression at the point in the shell where it will eventually be formed, and, also, with the secretion of the exochorion layers by the follicle cells. It has already been pointed out by Beament (1946*b*) that this relationship exists in the less specialized portions of the chorion. In the main part of the shell, undulation of the endochorion surface produces regular depressions at the bases of the follicular pits, but the folds which precede the secretion of the rims of the shell and cap are themselves depressions in the substrate on to which secretion takes place. On the other hand, these folds also produce convexities which appear to have a retarding effect on the production of pits. It was found (Beament, 1946*b*) that pit formation was also slightly retarded over the convexity of the rear end of the shell.

It is therefore interesting to note that the deepest pit in the cap, that formed by the *A* cells, is formed in the anterior depression of the initial fold, and that it lies entirely within the exochorion, which is very thick at this point. However, the *B* cells, secreting over the convex portion of the fold, produce a relatively short pit, while the *C*, *D*, *F* and *H* cells do not form any kind of pit. In the other depression, opposite the *K* cells, the micropyles and pseudomicropyles are formed. While both these types of 'pit' start in the endochorion layers, and so form exceptions to the correlation mentioned above, it may also be pointed out that in the shell units which contain these specialized cavities, three of the endochorion layers are missing, and so the formation of the pits may be aided by the transition of the cells to exochorion formation at a very early stage in shell secretion.

SUMMARY

An investigation has been made of the junction between the shell and cap in the egg-shell of *Rhodnius prolixus*. This complex region consists of the thickened rim of the cap connected by a thin sealing bar to the rim of the shell. The secretion of this part of the shell has been followed and compared with the formation of less specialized portions of the shell.

The shell has been divided into units, each the product of an individual follicle cell. It has been found that all the seven layers which make up the unspecialized

parts of the shell are present in the seal complex; that these consist of five endochorion layers and two exochorion layers in their normal order.

The exochorion is secreted around long villi, one from each follicle cell. These give rise to follicular pits in the shell.

In this complex region, cells start to secrete at various stages in the seven-phase cycle; their initial secretion is apparently related to the material with which they make contact at that time. After secretion has started, each cell completes the remainder of the cycle.

The rim of the cap is the product of four rings of follicle cells; the additional thickness is achieved by an increase in the exochorion layers, secreted around a series of very long follicular pits.

The sealing bar, which is produced by one ring of follicle cells, is composed of the inner four layers of the chorion only; the cells do not produce soft endochorion, or exochorion layers.

At the cap end of the sealing bar there is the predetermined hatching line. It is apparently produced by the presence in the follicle of cells which are inactive during the secretion of the inner layers, and so prevent co-ordination between the active cells on either side. A weak point is also present at the base of the sealing bar, at the site of other inactive cells, though this fissure is not used at hatching.

The rim of the shell is similarly produced by an expansion of the exochorion layers secreted around four rings of follicular villi. Of these, three rings of pits are filled in towards the end of secretion, but the fourth, lying on the upper portion of the rim, remains. These pits become the micropyles and associated structures.

There are 200 pits in the completed rim, divided into two groups. About fifteen are micropyles; the remainder are cavities closed at each end, and to which the name 'pseudomicropyle' has been given.

The pseudomicropyles are formed in a similar way to normal follicular pits, but start in the resistant protein layer, $0{\cdot}5\mu$ from the inside of the shell. They end in the resistant exochorion, where they are connected to the external surface by small bunches of pore canals. They probably play some part in the respiration of the embryo.

The true micropyles form the only free path through the shell. The inner portion of each tube is lined with hydrophilic protein, and the outer portion, which lies slightly posterior to the pseudomicropyles, is composed of hydrophobic lipoprotein.

The number of true micropyles is not constant, there being between ten and twenty scattered irregularly around the rim. However, eggs produced by older females contain fewer micropyles; this may account for a higher rate of sterility among these eggs.

The cells which form the micropyles and pseudomicropyles are the only ones which do not adhere to the typical cycle of seven secretory products. But in omitting three phases, the attachment of the exochorion to a protein layer is retained.

Evidence suggests that the cells forming the micropyles are determined in the earliest stages of secretion by being squeezed out of the pseudomicropylar ring of cells.

I wish to express my gratitude to Dr V. B. Wigglesworth, F.R.S., for his help and advice during this work. Thanks are also due to Miss W. Wall and to Mr J. H. Birch (of the London School of Hygiene and Tropical Medicine) for their technical assistance.

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THE BEHAVIOUR OF WIREWORMS IN RESPONSE TO CHEMICAL STIMULATION

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(With Plate 9 and Eleven Text-figures)

I. INTRODUCTION

If wireworms are ever to be controlled by trapping it is necessary first to discover how they find their normal food under natural conditions. The handicap created by the scarcity of laboratory experiment on the food finding and feeding behaviour of wireworms (*Agriotes sputator*, *obscurus*, *lineatus*) must have been obvious to all those who have tried to devise a practicable field method of control by trapping. Without such basic laboratory observation properly co-ordinated with field experiment there is little prospect of knowing whether or not baiting can be a practical technique of control, and all attempts to elaborate an efficient method are bound to be empirical. It was for this reason that the present work was undertaken.

The periodic movements of wireworms in soil are of course vitally important in relation to the problem of food finding. It has long been realized that wireworms tend generally to move downwards in the soil during the late autumn and to some extent during periods of summer heat and drought and upwards again in spring and late summer. It has recently been shown that such movements are probably based on orthokinesis, and on a 'shock reaction' to temperature extremes and to humidities below saturation and are not brought about by any response to gravity—such a sense being completely lacking to the wireworms when burrowing in soil (Lees, 1943*a*, *b*; Falconer, 1945*a*).

It is difficult to evaluate field observations on the efficiency with which wireworms find their food in the soil. In grassland there is clearly no problem. Here most individuals probably live in semi-permanent systems of burrows and remain fairly near the surface. Those that go down deeper in winter could hardly fail on rising in spring from the lower levels of the soil, to strike grass roots on which to feed. But under arable conditions things may be very different. While wireworms here, too, form their semi-permanent systems of burrows (Lees, 1943*b*), used once they have found their food, they certainly tend to descend more deeply and uniformly in winter, and during this period their burrows will soon get destroyed by cultivation and the effects of weather. Obviously, if when they rise again in spring a large proportion of the surface is covered with crop plants or if there are well-developed

root systems, the wireworms will have little difficulty, merely by random burrowing in the soil, in finding something to eat. But though under such conditions they may find food in the form of fine rootlets, this, since the larvae beneath the soil lack all guidance from light and gravity, might aid them little in finding the succulent central parts of the plant. Now it is clear that the efficiency of food finding of wireworms does vary with environmental factors such as soil texture and method of cultivation. Nevertheless, one cannot fail to be impressed by the speed and apparent certainty with which, at any rate in some instances, even quite small plants can be located in spring. Erratic as the results of experiments with trap crops are apt to be, they do provide some evidence of considerable powers of orientation to food, at any rate over short distances (Miles & Petherbridge, 1927; Ladell, 1938; Petherbridge, 1938), and the records of the rapid and very high infestation of potato tubers by reasonably small populations of wireworms (*Bull. Minist. Agric.* no. 128, 1944, pp. 44–5) also point to this conclusion. It seemed possible, therefore, that a chemical method of orientation might be involved. Preliminary experiments by one of the authors (W.H.T.) had been successful in showing that responses of wireworms to plant extracts and to individual chemical substances could be studied conveniently in the laboratory. The object of the present paper is to give an account of that part of the work which was primarily directed to answering the question—'How do wireworms find their food in the soil?' The greater part of the laboratory work here described has been carried out by two of us (A.C.C. and J.H.D.). Further papers on the relation between activity and chemical constitution (A.C.C. and J.H.D.) and on the sensory equipment of the wireworm (A.C.C.) are in preparation.

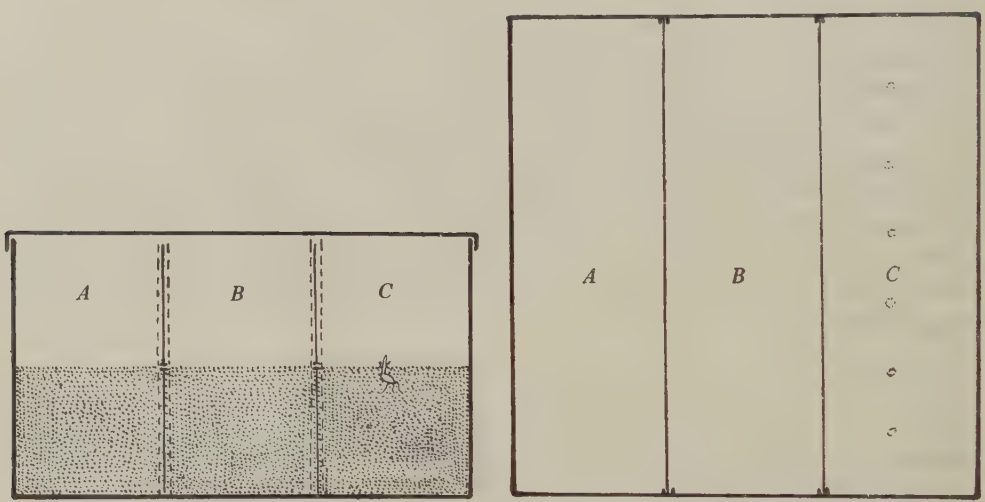
II. MATERIAL AND METHODS

Source of material for experiment

Because of the difficulty of keeping large stocks in healthy condition in the laboratory for any considerable period we decided to rely on material freshly collected from the field. During the first 2 years it was possible to obtain large samples from farmers and from the various wireworms survey teams of the Ministry of Agriculture. Later, owing to the development of mechanical methods of sorting soil samples, the latter source dried up almost completely, and it was soon found that the careless handling and delays inseparable from the former method might involve such a large mortality or, at best, loss of condition, that it was best avoided. Accordingly for the greater part of the time high wireworm infestations in the neighbouring districts of Cambridgeshire were kept under regular observation, and our material directly collected by ourselves as and when soil and cropping conditions made the extraction of large numbers feasible. For help in finding suitable infestations we are indebted to Mr F. R. Petherbridge, Cambridge School of Agriculture, and his assistants of the advisory staff; to the officers of the Cambridge War Agricultural Executive Committee; and among farmers particularly to Mr W. Jackson of Hill Farm, Upware, whose success in overcoming by good cultivation and management a high wireworm infestation on what was previously semi-derelict fenland, has been remarkable. We

are also greatly indebted to Mr Percy Hardwick of Fulbourn, to Mr G. W. J. Burden of Hardwick, and to Mr Hawkes of Barton, as well as to Mr Jackson, for providing invaluable facilities for field experiment, and for much co-operation and forbearance without which the work could not have been completed.

Like all other workers with wireworms we have had to accept the present impossibility of distinguishing between *A. lineatus* and *A. obscurus* in the larval stages. Thus our material of these two species may have been either one or other or mixed populations of both. Whenever we have been able to obtain numbers of *A. sputator* we have, as is made clear below, used this as well. Our results have given no indication of there being any substantial difference in behaviour between *sputator* and *lineatus-obscurus* populations. We therefore assume for our present purpose that the three species are identical in food-finding responses. There does not at present seem to be any definite indication from the field against this assumption.



Text-fig. 1. Container with sand divided into three sections by partitions in air and sand. Wheat growing in section C (see text).

Many previous workers have found that populations of wireworms show two moderately well-defined non-feeding periods each year, one during the winter and the other in the early summer. Conversely, there are peaks of feeding activity in spring and autumn. The fasting periods are no doubt in part an expression of the vertical soil movements which are governed by temperature and humidity changes (Lees, 1943*a*, *b*; Falconer, 1945*a*), but it has been shown by the study of individual wireworms (Evans, 1944) that each moult is followed by a feeding phase of a few weeks duration followed by a slightly longer fasting phase. Larger wireworms moult less frequently than smaller and tend to remain longer in the fasting phases. Thus the feeding behaviour of a population is largely the expression of the heterogeneous physiological rhythms of its constituents. As it is more convenient to use the larger wireworms for experiment, the dependence of feeding period on the

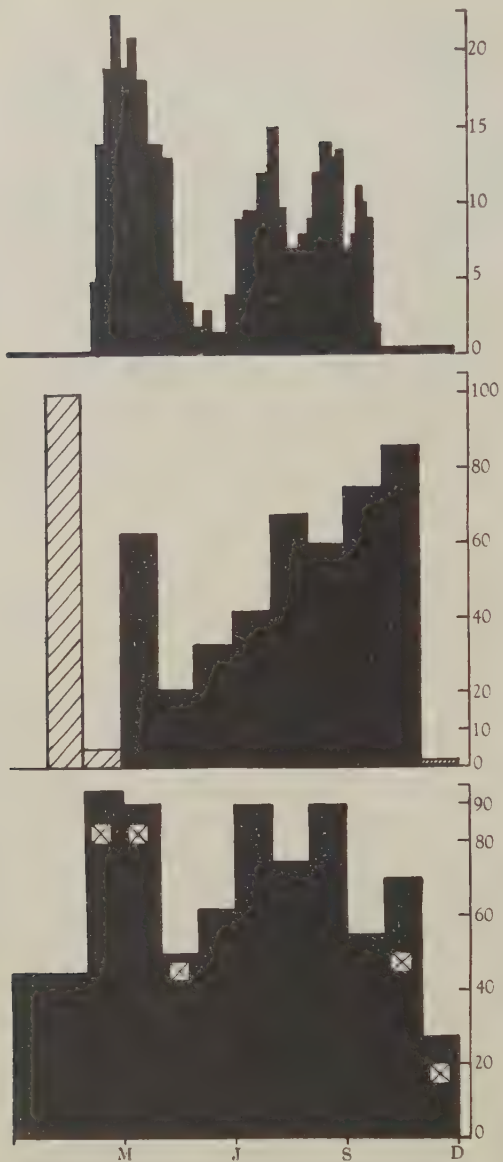
size raises a difficulty. It was found that while the biennial rhythm may be modified by transference from field to laboratory conditions, the fasting periods cannot be eliminated thus. The seasonal variation in the percentage of wireworms giving the orientating response in routine tests throughout 1943 and 1944 is shown in Text-fig. 2. This shows how far active and inactive periods correspond to the feeding and non-feeding periods of Evans (1944). It also shows how during certain times of the year experiments may be impossible owing to the high proportion of non-feeding individuals in a population. Raising the temperature of the stocks from 16 to 23° C. or subjecting them to a preliminary cold shock at 5° C. for 2 days did not significantly increase the number responding. All batches of wireworms used for orientation experiments were tested against a standard 'bait' of 2% sucrose to determine their activity. Later, when a technique for studying the behaviour of single wireworms in the plate apparatus was perfected (see below), these difficulties were avoided by selecting only active wireworms. All the larvae used in these experiments were from 1·5 to 2·0 cm. long, representing the later instars (Salt & Hollick, 1944).

Methods and apparatus

All experiments with the apparatus here described were performed in a constant temperature room at either 16 or 23° C.

(*a*) *Testing responses when in air.* To test the responses to airborne odour an ordinary glass Y-tube olfactometer 5 cm. in internal diameter and filled with glass beads to provide the necessary tactile stimulation was first employed. Wireworms showed no response to odours in this apparatus. The apparatus, however, is inefficient for a relatively slow-moving soil-dwelling animal. The area of contact between the two air streams to be tested is small, and the light beam necessary to ensure that the animals travel up the tube to the junction introduces an unnatural factor into the situation. To avoid some of these difficulties a choice chamber was devised.

This second apparatus was a modification of that described by Wigglesworth (1941). Into a deep Petri dish across which a watertight partition had been fixed were introduced, on opposite sides of the partition, equal volumes of distilled water and the solution to be tested. The surfaces of the liquids on the two sides of the partition were at the same level. A metal cylinder, 1 in. deep, across one end of which a piece of fine brass gauze had been fixed, was now placed, gauze downwards, into the Petri dish. Supports were arranged so that the gauze was a few millimetres above the surface of the liquid. Beads were now placed in the space above the gauze, and a number of wireworms placed on top of the beads in the centre of the cylinder. The whole was now lowered into a deep, blacked-out water-bath (maintained at 2° C. above air temperature) so that the bottom of the Petri dish was supported below the surface of the warmed water. The space above the water in the water-bath was saturated with water vapour. Vapour from the distilled water and the odorous substance rises up through the gauze and the beads, and the wireworms above the gauze would show a response to the odour concerned by aggregating on



1942

Seasonal variation in activity of a group of 175 wireworms under out-door conditions as measured by units of food (wheat grains) consumed weekly.
(A. C. Evans's data.)

1943

Seasonal variation in percentage of wireworm population giving the aggregation response at 16° C. in laboratory. Cross-hatching represents results based on less than 5 experiments.

1944

Seasonal variation in percentage of wireworm population giving the aggregation response at 16° C. and 23° C. in the laboratory. (× = results at 23° C.)

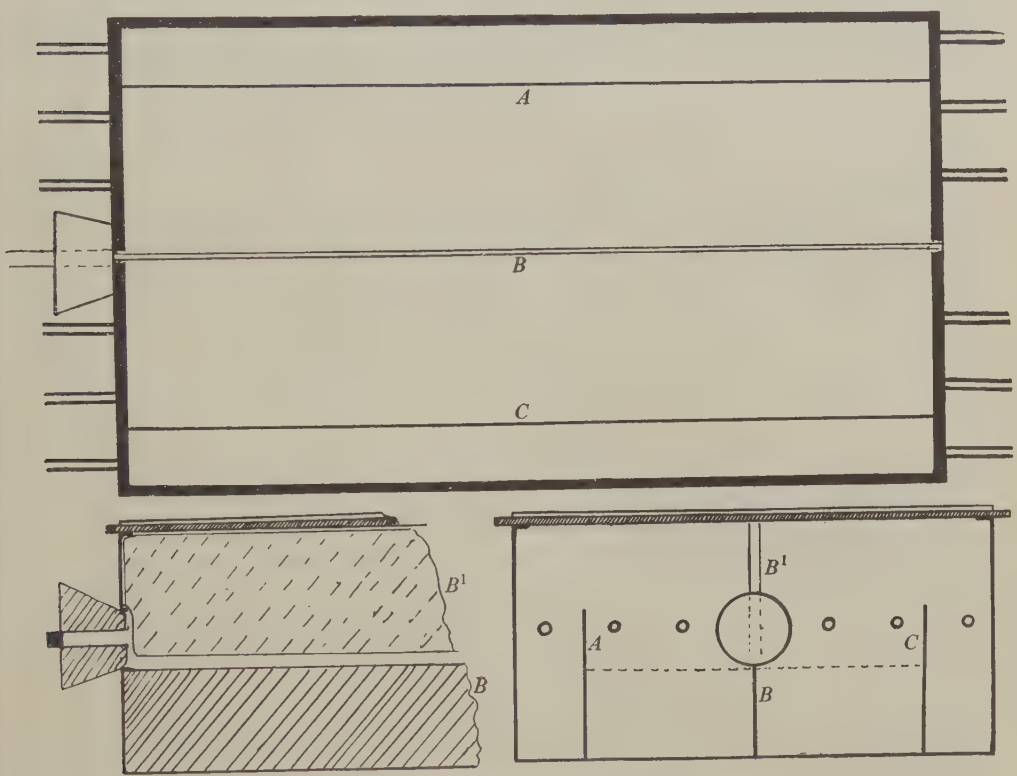
Text-fig. 2. 1942: seasonal variation in activity of a group of 175 wireworms under outdoor conditions as measured by units of food (wheat grains) consumed weekly (Evans's 1944 data). 1943 and 1944: seasonal variation in percentage of wireworm population aggregating in the laboratory; the number of days per month on which wireworms of each lot collected were active expressed as a percentage of the total number of lot-days. Cross-hatching represents results based on less than five observations. × indicates results obtained at 23° C., the others being at 16° C.

one side or the other of the partition. The numbers on either side were counted after 24 hr. In this apparatus, likewise, no response to odours was observed.

These negative results might have been due to the unnatural conditions in the two preceding types of apparatus preventing the wireworms responding normally to

chemical substances borne in the air. A third apparatus was therefore designed in which it was hoped that conditions would be more natural.

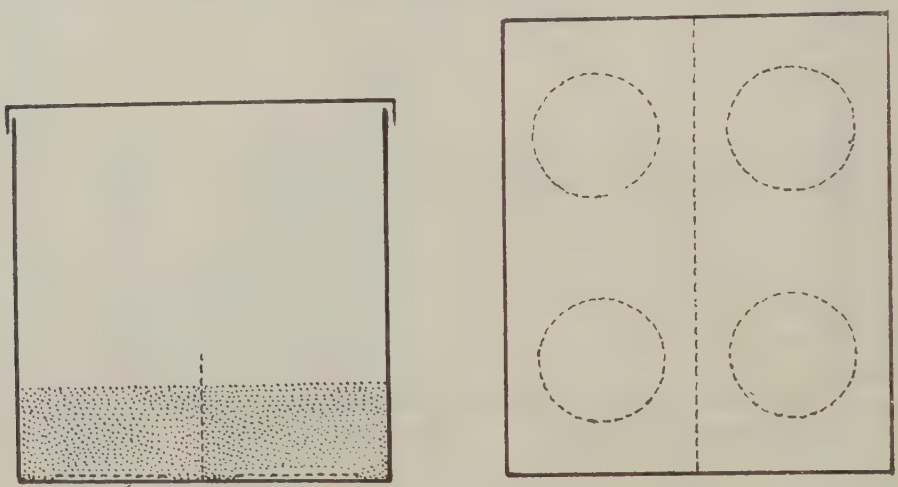
The plan and elevation of this apparatus, which was constructed of metal, are shown in the accompanying diagram (Text-fig. 3). The damp sand in the central space just covers partition *B*, so that an unbroken surface of sand stretches from *A* to *C*. The glass partition B^1 which divides the air space into two compartments comes down to within about 5 mm. of the surface of the sand, so that a wireworm can easily pass under it. The glass lid is made airtight with a rubber gasket. The



Text-fig. 3. Plan and elevation of olfactometer (see text).

'bait' solution is either placed in one of the side troughs divided off by partition *A* or *C* (the other containing an equal volume of distilled water) or air is bubbled through a 'bait' bottle before entering the inlet tubes on one side as was done with the Y-tube apparatus, or finally the bait may be placed in both positions. Wireworms entering the apparatus at one end below the central glass partition encounter the junction between the two streams of air, and after wandering about on the surface of the damp sand, eventually burrow down into it. A response to the odour concerned would be shown by their aggregation on one side or the other of the central partition (§ IV (ii) (*a*)).

(*b*) *Testing responses to dissolved substances when in soil.* The responses of wireworms in sand or soil were investigated by two methods. The first made use of a metal box 13 × 19 cm. and 15 cm. deep with a movable metal or glass partition dividing it lengthwise into two (Text-fig. 4). Washed sand to a depth of 3·25 cm. (800 ml.) was placed in the bottom. The sand on one side was moistened by thoroughly mixing with 5 ml. of water, that on the other with the same quantity of solution to be tested. To start the experiment the partition was removed and the wireworms placed in the middle region of the sand surface. At the end of the experiment the numbers in the 'baited' half and the unbaited half of the apparatus were counted. This choice chamber thus tests the ability of the insects to orientate in sand or soil in solutions of a given substance. All 'aggregation experiments' were performed at 16° C.,



Text-fig. 4. Plan and elevation of sand-choice chamber. The broken lines at the bottom of the sand in the elevation and the circles in the plan represent filter papers used when testing the biting response (see text).

except when attempting to break dormancy when they were performed at 23° C. (see above). There was no observable difference in responses given at the two temperatures.

The second method makes use of the fact that wireworms will bite filter paper soaked in solutions of certain food substances and their bite marks can easily be counted, thus providing, when compared with a control set of filter papers moistened with water, a numerical measure of the 'palatability' of a given substance. Individual wireworms may vary considerably in the number of bites given on active substances; for this reason they were used in groups of 20. The amount of biting may be the index not only of palatability but of feeding activity. The latter was therefore standardized by measuring the amount of biting elicited by a standard solution (2% glucose) from samples of each group of wireworms collected. Most groups gave 150–600 (average 350) bites per 20 wireworms in 24 hr., those biting beyond these limits being rejected for subsequent tests. This represents the usual

amount of biting and degree of variation of most of the groups collected (see discussion on Text-fig. 6). Wireworms bite the filter papers impregnated with an active substance and also the controls containing water. The amount of biting on the latter is rather variable and bears no relation to the amount falling on the baits, but does not exceed about 70, that is, 20% of the total number of bites given in positive tests. This represents the residuum of biting not chemically stimulated. Because of this variability the controls were ignored when estimating 'palatability', the total number of bites per 20 wireworms in 24 hr. being taken as the index of this factor. As a qualitative test the method was also useful in determining whether some of the less palatable substances were palatable or not: the bites on filter papers with palatable substances are characteristically close together (Pl. 9). On the controls they are distributed evenly over the filter paper.

A large number of experiments with baited filter papers were carried out in an apparatus consisting of two sheets of plate glass a foot square separated by a marginal rubber gasket 5 mm. thick and held in a wooden frame. The lower glass sheet is covered with a layer of paraffin wax 1 mm. thick in which are eight circular cavities, 5 cm. in diameter, arranged in a ring. A piece of filter paper (5 cm. in diameter) is fitted into each of the eight cavities. Four alternate filter papers are moistened with five drops (0·25 ml.) of the solution to be tested; the other four with the same quantity of water. The apparatus is then filled with glass beads in a saturated or nearly saturated atmosphere, 20 wireworms introduced, and the top sheet put into position. After 24 hr. in darkness the filter papers are removed and the number of bites on each counted under a binocular microscope.

It was found that baited filter papers could be used equally well under damp sand in the bottom of the choice chamber box as shown in Text-fig. 4, and this has the advantage of avoiding any difficulty due to an unsaturated atmosphere. This was the method generally adopted in later experiments. The sand was passed through sieves, and only grains between 0·1 and 0·2 mm. in diameter were used in this and other apparatus described in this paper. The sand was 14·3% saturated with water. The value for saturation was obtained by adding water to the sand in a measuring cylinder until free water just appeared at the surface (Lees, 1943*b*). The sand was 14·3% saturated when 5 c.c. water were added to 400 c.c. sand. Both biting and orientation took place best with this amount of moisture. This is shown in Table 1. In 28·6% saturated sand there was practically no biting and the wireworms were too sluggish to move. The 5·7% saturated sand, on the other hand, was much too dry; the wireworms gathered on the wet filter papers or huddled together in the corners of the apparatus. But biting does occur even at this degree of saturation: in one experiment in which the wireworms gathered on a sucrose filter paper, this was closely bitten. In the 14·3% saturated sand the wireworms exhibit both the normal orientation and biting responses. All 'biting experiments' were performed at 23° C. The biting response is exhibited at 16° C., but the number of bites given is less.

(*c*) *Apparatus for observing tracks in sand or soil.* The mechanism of orientation of wireworms to solutions in soil was examined by means of an apparatus similar to that used by Lees (1943*b*) and Falconer (1945*a*) for observing their tracks in sand or soil.

This apparatus consists of a glass plate to which are cemented glass strips 12 cm. long and 2 mm. deep forming a series of square compartments. Each compartment is marked into two by a line scratched across the middle. When testing responses in soil, each compartment is filled with sieved air-dried fen soil which is rolled smooth and level with the top of the glass strips. The soil may then be impregnated with suitable amounts of solutions of various substances by spraying from a fine pipette either the

Table 1. *The effect of moisture on biting* (20 A. obscurus-lineatus, 24 *hr.*) *35 c.c. water saturates 100 c.c. sand*

No. c.c. water per 400 c.c. sand	% saturation of sand	No. exps.	Total no. bites on filter paper with	
			Water	2% sucrose
2	5·7	4	55	75
5	14·3	2	25	580
10	28·6	2	0	45

Table 2. *Responses to growing wheat.* A. obscurus-lineatus *were released in B* (*Text-fig.* 1). *Wheat was growing in C. The numbers in sections A, B and C respectively, were counted after* 48 *hr.* (23° C.)

	No. wireworms per section			Total no. migrating to A and C	No. in C as percentage of total no. migrating
	A	B	C		
(*a*) No partitions	8	6	49		
	17	15	97		
Total	25	21	146	171	85·5
(*b*) Partitions in air only	14	50	59		
	27	30	44		
	23	79	85		
Total	64	159	188	252	74·9
(*c*) Partitions in sand only	20	14	76		
	23	47	92		
Total	43	61	168	211	79·6

whole surface of each compartment or, if a boundary is desired, half of its surface with the solution and half with the same amount of water. After the liquids have soaked in, the surface is rolled again and a wireworm placed in the required position; each compartment is then covered with a glass plate. It can be seen that the animals burrow by pushing aside the fine soil and by picking up the larger crumbs and grains in their mandibles and placing them on one side. (E. T. Burtt unpublished MS.) The tracks thus made are traced after a suitable lapse of time, usually two hours. When testing responses in sand the procedure is similar, but the water and solution, respectively, are mixed with the sand before the latter is placed in the compartment. The tracks made in sand may be most readily seen by illuminating the apparatus from below. The experiments were performed in darkness at 16° C. The sand used consisted of particles between 1 and 2 mm. in diameter. The soil was the fraction

that passed through sieves with 8 meshes to the inch, but was retained in sieves with 30 meshes to the inch.

III. FINDING OF LIVING PLANTS BY WIREWORMS: PRELIMINARY LABORATORY EXPERIMENTS

That wireworms are able to find growing wheat either by moving through the soil or by coming to the surface and moving over it was shown by the following experiment (cf. Falconer, 1945*b*). Wheat was grown in a row in clean sand in one section (C) of the container shown in Fig. 1. Wireworms were released in section *B* which was separated from A and C by two sets of partitions, one in the air reaching to the surface of the sand and the other reaching from the bottom of the air partition to the bottom of the container. When the wireworms had burrowed beneath the sand, in different experiments either all the partitions or only those in the sand or in the air were removed. The sand in the three sections was sieved after 48 hr. and the wireworms counted. The results (Table 2) show that wireworms can move both through the sand and over its surface, and that, in either event, more collect in section C containing the wheat than in section A containing none.

It was originally intended to investigate how far the apparent food preferences exhibited by wireworms in the field are the expression of selective response to differences in chemical composition. But the field evidence for food-plant preference is very confusing, since all that is usually recorded is the number or proportion of plants destroyed or showing injury (cf. Subklew, 1934). There is thus no means of allowing accurately for the fact that some resistant crops (e.g. potato) can survive a considerable attack without showing much damage above ground, whereas others (e.g. freshly germinated cereal) may be destroyed by the first bite. Evans (1944) showed flax to be physiologically unsatisfactory as a nutritive material for the wireworm, but filter papers impregnated with the juice of its roots were bitten (§ IV (i) (*a*)), and there seems to be no real evidence for assuming that this or other so-called 'resistant' crops are in any degree unattractive or unpalatable to wireworms. Most so-called resistant crops are probably not repellent or immune from attack to any extent but are merely more able to recover from it when it occurs. For this reason and also because of the complexity of the chemical problem we did not proceed with this part of the work.

IV. RESPONSES TO PLANT EXTRACTS AND CONSTITUENTS

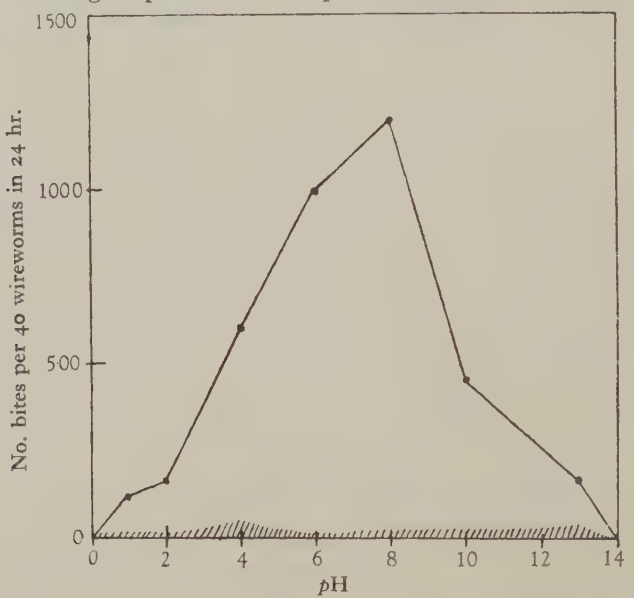
(i) *The biting response*

(*a*) *Plant extracts causing biting.* The biting response of fed wireworms is elicited by the juice of potato tubers, carrot and sugar-beet tap roots and wheat and flax roots, and by an aqueous extract of wheat bran. The extract was placed on filter papers in the biting apparatus described above. The first problem was to discover the active chemical substance in these extracts.

The activity of substances causing either of the responses is defined as follows: If 1 g. of a substance is dissolved in x ml. of water to reach the threshold, then the

activity of that substance is defined as $\log_{10} x$. For example, a substance for which the threshold is a 1% solution has an activity of 2. The threshold is defined as the lowest concentration which causes any response.

Among other substances, the sugars glucose, fructose, and sucrose (Table 3) are active in concentrations at which they are present in the plants. For instance, with fed wireworms the palatability of sucrose falls from the maximum to zero between 0·5 and 0·2%, that of glucose between 1·26 and 0·5% (Text-fig. 6). Expressed potato juice, which contains about 1% glucose and 0·1% sucrose, is active but becomes inactive when diluted 10 times. Sugar-beet juice, which contains about 18% sugars, is active when diluted 10 but not 100 times. An extract of bran with 10 times its weight of water contains 0·4–0·6% sugars. It is clearly the sugars which elicit the biting response to these plant extracts.



Text-fig. 5. Relation between *p*H and number of bites given per 40 wireworms in 24 hr. on 2% glucose. Shading represents maximum biting on the water control filter papers.

The effect of *p*H on biting was tested as follows. 2% glucose solutions were buffered at different *p*H values from 0 to 14. The buffers themselves were previously tested and found inactive. The following buffers were used to obtain the *p*H values shown (Clark, 1928):

Buffer	*p*H	Buffer	*p*H
N HCl	0	Sorensen	6–8
N/10 HCl	1	*N*/30 KH_2PO_4-Na_2HPO_4	
Clark and Lubs	2–4	Carbonate-bicarbonate	10
N/20 phthalate HCl		Glycine-NaOH	12
Acetic	4–6	*N*/10 NaOH	13
Sodium acetate		*N* NaOH	14

The wireworms were starved for 1 week before the experiment. When the total number of bites given by 40 wireworms on filter papers soaked in glucose solution is plotted against the *p*H of the solution, the curve shown in Text-fig. 5 is obtained.

Maximum biting occurs between *p*H 6 and 8, and biting decreases as the solutions become more acid or more alkaline. The number of bites on the controls varied between 0 and 40. Most plant juices have a *p*H of 5–7.

(*b*) *Chemical substances causing biting.* A list of the chemical substances (2% aqueous solutions unless otherwise indicated) tested for the biting response is given in Table 3. Two or more experiments were performed with each substance. The

Table 3. *Substances tested for eliciting the biting response*

Carbohydrates, etc.

Active	Inactive	Active	Inactive
*Arabinose	Xylose	Sorbitol	Saccharin
Glucose	Lactose	*Mannitol	Glycol 5%
Fructose	Inulin	*Gluconic acid	Glycerol
Galactose	Starch		Glucose 1 phosphate
*Mannose			β-Pentacetyl glucose
Sucrose			
Maltose			
Stachyose			
*Dextrin			

Fats and fatty acids

Active	Inactive
Triolein 1%	Tripalmitin
Linseed oil	Palmitic acid
Olive oil	Tristerin
Wheat germ oil 6%	Stearic acid
Maize germ oil	Oleic acid
Lecithin	Na oleate
Na palmitate	Ethyl butyrate
Na stearate	Cholesterol
	Lanolin
	Clove oil
	Cooking fat
	Margarine
	Butter

Proteins, etc.

Active	Inactive	Inactive
Casein	Potato protein	9 amino acids
*Hen's egg albumen	Gliadin	2 protein hydrolysates
*Horse-serum albumen	Gelatin	(see below)
*Haemoglobin	Fibrinogen	
Dialysed tryptic digest of potato protein		
Peptone (B.D.H.)		

Miscellaneous

Active	Inactive
Tannic acid 1%	Acetic, valeric, oxalic, succinic, glutaric, maleic, tartaric and citric acids
Na tannate	Arginine, asparagine, cystine, glutamic acid, glutamine, glycine, leucine, tryptophan, tyrosine, acid hydrolysates of casein and potato protein
(Baird & Tatlock— a gallotannin)	Acetamide, amyl alcohol, agar agar, ammonia, ascorbic acid, methylamine, nuclein, phloroglucinol, propionamide, quinine sulphate, saliva, sodium chloride, sodium salicylate, urea, uric acid, vanillin

The substances were tested as 2% solutions or, in the case of fats and fatty acids, emulsions, except where otherwise indicated. Active compounds gave from 150 to 600 bites per 20 wireworms at 23° C. Those compounds marked with an asterisk, though definitely active, gave only 50–200 bites under the same conditions.

wireworms were starved for 1 week. The average number of bites elicited by most of the active substances was about 350 per 20 wireworms in 24 hr., with a possible variation with each substance from 150 to 600. The substances marked with an

asterisk were less palatable. Some of the latter, viz. arabinose, dextrin, hen's egg albumen, horse-serum albumen, haemoglobin, tannic acid and sodium tannate, sometimes elicited as many bites as members of the most palatable group, but the number elicited was usually between 50 and 200, and was occasionally no greater than that on the controls. Mannose, mannitol and gluconic acid only occasionally elicited more bites than the controls, up to 120 per 20 wireworms in 24 hr. There is, however, no doubt that these three substances are feebly active: this is indicated not only by the number of bites but by the characteristic close biting on the filter papers containing them as compared with the controls. These 'less palatable' substances did not elicit a greater number of bites at higher concentrations, e.g. in 5% solutions. Arabinose, glucose, mannitol, triolein, sodium stearate, casein, peptone and tannic acid were tested with *A. sputator* as well as *A. obscurus-lineatus*, with which all the substances were tested. No differences in biting behaviour were observed between wireworms of the different species.

The compounds which cause biting are all members of the three major food groups, carbohydrates, fats and proteins, and are tabulated in this grouping in Table 3. Tannic acid is an exception and gives a weak response.

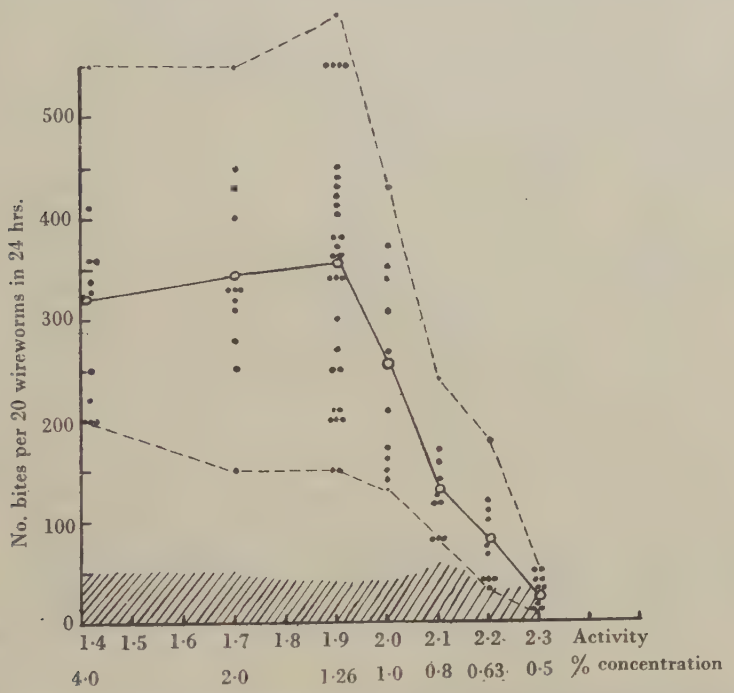
In the carbohydrate group not only are the common plant sugars—glucose, fructose and sucrose—highly active but also other sugars ranging from mono- to tetrasaccharides and dextrin. Solutions of starch and inulin do not cause biting. In sorbitol, mannitol and gluconic acid the aldehyde group has been altered and the ring structure destroyed but the compounds remain active. This suggests that the polyhydric alcohol grouping is responsible for the activity as in the case of the sweet taste in man. In the biting response there is, relative to human taste, a shift towards larger numbers of alcohol groups as the wireworm is sensitive to dextrin but not to glycol or glycerol. This resemblance to the sweet taste is confined to polyhydric alcohols, for glycine and saccharin cause no response.

Fats have been tested both as emulsions stabilized with sodium oleate or cetyl trimethyl ammonium bromide and by depositing weighed amounts on filter papers from a solution in ether. The latter method is preferred as it is less variable, but the compounds shown in the table have been tested as 1% emulsions except where the contrary is stated. Triolein is the only pure fat found to be active, but as linseed oil contains less than 5% of its fatty acids as oleic acid (Cocchinaras, 1932) the glycerides of either of its other major component acids, linolic and linolenic, may be active. Tristearin and tripalmitin are not active, neither is glycerol, but these two fatty acids are active as sodium salts. Wheat-germ oil is active as a 6% emulsion but not at 2%. It contains 28% of its fatty acids as oleic (Jamieson & Baughman, 1932).

The proteins so far found to be active are of animal origin, while the plant proteins tested are inactive. Partially broken-down proteins may be active though the parent proteins were not, but none of the amino acids or mixtures of amino acids tested have proved active.

(*c*) *Starvation and the threshold.* The activity of all the substances shown in Table 3 is at least 1·7 for wireworms starved for 1 week, i.e. 2% solutions are active. For fed wireworms the activity of sucrose was 2·7 and that of peptone 2 to the nearest

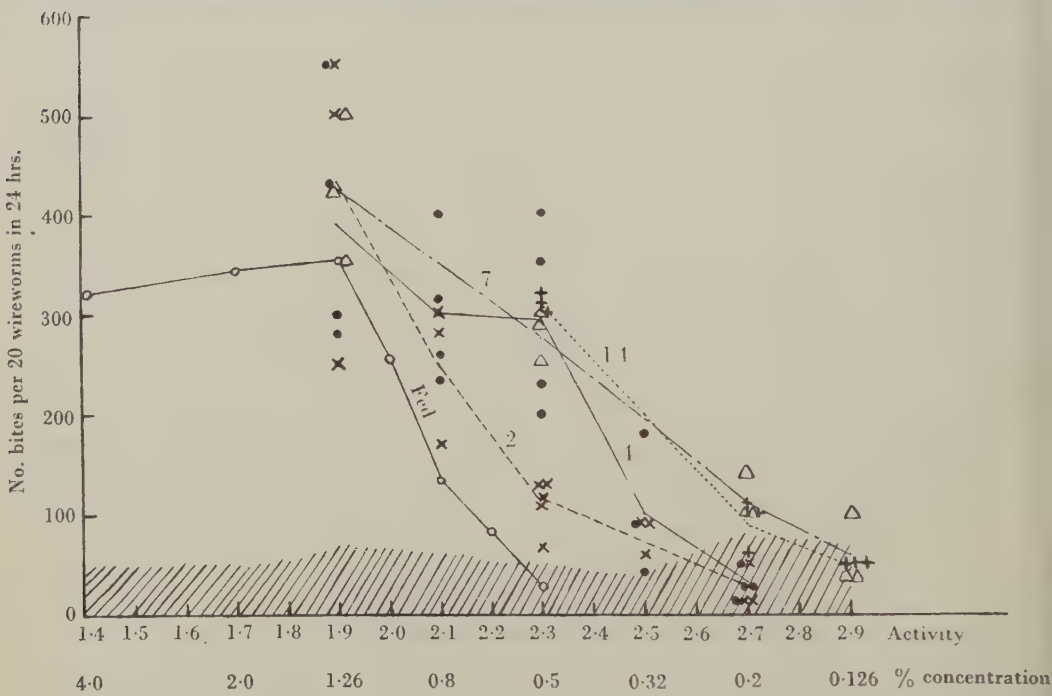
0·2 log unit, while the threshold for triolein was between 17 and 15 mg. per filter paper (5 cm. diam.) or between 1 and 0·1% emulsion. The activity of glucose was more precisely determined with fed wireworms of the species *A. obscurus-lineatus*, all collected from the same place and of standard feeding activity. They had been fed on potato slices up to the beginning of the experiment. The results are shown in Text-fig. 6, in which the activity corresponding to each value for the concentration of glucose is shown on the abscissa. The average number of bites elicited per 20 wireworms in 24 hr. decreased from a maximum of about 350 when the concentration fell below 1·26% and reached a value no greater than that on the control filter



Text-fig. 6. Relation between concentration of glucose and number of bites given per 20 wireworms in 24 hr. Dots represent individual results, the broken line the limits of variation, and circles and the full line the average number of bites on the glucose. Shading represents maximum biting on the water-control papers.

papers at 0·5%. The variation in the number of bites is considerable at the higher concentrations, but decreases with concentrations below 1·26%. The same wireworms were now starved and their sensitivity to glucose measured after different intervals. Time is measured from the last feed to the beginning of each test. As shown in Text-fig. 7, up to 7 days the animals become more sensitive to glucose solutions as starvation increases. Starvation beyond 7 days has no further effect on the threshold. The lowest concentration persistently eliciting a response is 0·2% corresponding to an activity of 2·7, although definite biting occasionally occurs at 0·126% (activity 2·9). It seems unlikely that the sense organs of fed wireworms were adapted at the beginning of the experiment to the sugars in the potato on which they

were fed, and that the fall in the threshold with starvation corresponded to the disappearance of adaptation. The time taken seems altogether too great for such a process. A more probable explanation is that the limit of sensitivity of the receptors is reached after 7 days' starvation, this not being realized in the behaviour of fed wireworms because some central inhibiting factor, whose effect decreases with increasing starvation, intervenes between stimulus and response. Starvation has a similar effect on the behaviour of various other insects, e.g. the red admiral butterfly, *Pyrameis atalanta* (Minnich, 1922), blowflies of the genus *Calliphora* (Minnich, 1929, 1931; Haslinger, 1935) and the honey bee (Minnich, 1932; von Frisch, 1934).



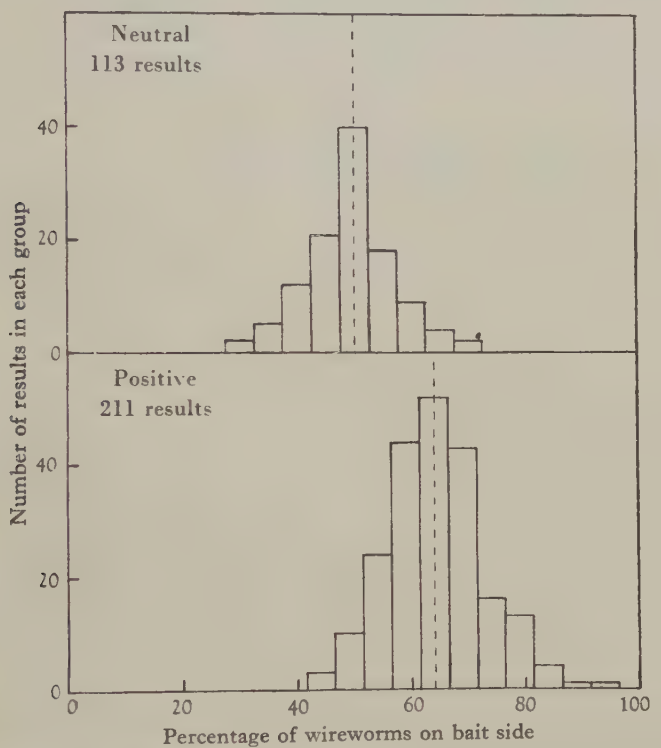
Text-fig. 7. Relation between concentration of glucose and number of bites given per 20 wireworms in 24 hr. and with different degrees of starvation. The lines connect the average numbers of bites on glucose of different concentrations after the number of days' starvation indicated by the figures against them. Individual results shown by × after 2 days' starvation, ● after 4 days, △ after 7 days and + after 14 days. Shading represents maximum biting on the water-control papers.

(ii) *Chemotaxic orientation*

(*a*) *Response to airborne odours.* The responses of wireworms to airborne odours were tested in the olfactometer described above (Fig. 3). No response was observed to air which had been bubbled through mashed up potatoes or carrots. A petrol (below 40° C.) extract of potato pulp was allowed to evaporate on cotton-wool. After the petrol ether had been removed there was no response to air passed through the cotton-wool. Wheat was grown in a shallow dish of damp sand till a mat of roots was formed. This was placed for 48 hr. while still growing on a pad of 10 g. of

cotton-wool containing 2 g. of lard evenly spread by evaporation from a solvent. The lard absorbed volatile compounds from the roots and the pad had a strong vanillin-like smell. Air saturated with water at room temperature was passed through the cotton-wool in a flask heated to 100° C. in a water-bath, and cooled before passing through the olfactometer. The wireworms did not respond, though sufficient volatile compounds were carried over for the human nose to distinguish between the two streams of air emerging.

(*b*) *Responses to dissolved substances.* When the aqueous extract or juice of flax seedlings, wheat bran, sugar beet, mangold or carrot tap roots, or potato tubers was



Text-fig. 8. Frequency diagram showing distribution of results of 324 experiments.

mixed with the sand on one side of the choice chamber described above (§ II) and the same amount of water with that on the other side, wireworms of all the species used orientate to the plant extract.

In the original orientation tests, any significant difference from 50% was decided by inspection; no statistical analysis was carried out. After a sufficient number of tests had been made, a statistical analysis was carried out on the whole of the results available. The percentage of wireworms found on the bait side was calculated for each of the three batches of about 100 wireworms used in testing one substance. The accompanying frequency diagrams (Text-fig. 8) show the numbers of experimental results occurring between limits of 5% for the substances classed as positive

and for the remaining tests, which are here called neutral. Tests may be neutral for two reasons: either the test solution or the wireworms may be inactive. The symmetry of the neutral diagram shows that the positive results have not been obtained by arbitrary selection of results greatly exceeding 50%. It also suggests that very few or none of the substances tested give negative (repellent) results, although many are positive and fewer neutral. The normal distribution cannot rigidly be applied as this case is limited in two directions, viz. 0 and 100%. In the neutral diagram, at least, the tails are far enough from 0 and 100% for an estimation of the standard deviation, considering the distribution to be normal, to be used as a test of the significance of any deviation from 50%. In fact, the third and fourth cumulants g_1 and g_2 for the neutral diagram are 0·013 and 0·309, and their standard errors are $\pm 0{\cdot}227$ and $\pm 0{\cdot}452$ respectively; for the positive diagram g_1 and g_2 are 0·400 and 0·381, and the standard errors are $\pm 0{\cdot}168$ and $\pm 0{\cdot}333$. This means that the neutral diagram is not significantly different from the normal distribution, and the difference of the positive diagram is only just significant (Fisher, 1934). For the neutral diagram, the standard deviation of one result in this test is 7·8%, and for the mean of three results the standard error is 4·5%. If the difference from 50% of the mean of three results is greater than twice the standard error the solution used as bait is considered to be active. The standard deviation for the positive diagram is 8·2%.

When the concentration of an active substance is increased above the threshold there seems to be no increase in the number of wireworms on the bait side. Neither is there any difference in the number collecting on the bait side in different active substances each above the threshold. These observations are borne out by the positive frequency distribution which is based on the response (as defined above) to many different substances and to concentrations of single active substances varying by as much as a factor of 10^8; yet the standard deviation is not significantly different from that of the neutral curve. Any active substance above the threshold causes 64% of the wireworms to collect on the bait side with random variations corresponding in magnitude to those which occur when no active substance is present. Another test of activity is whether wireworms respond to a substance in the plate apparatus (§ III (*d*)). Tests with individual wireworms in this apparatus show that many of them are inactive and that active individuals may wander about on both sides of the boundary between substance and water. These facts may explain the low proportion collecting on the bait side in the choice chamber.

Orientation is brought about by sugars (Table 4), their activity being between 2 and 3 for fed wireworms, and, contrary to what was stated in our letter to *Nature* (Thorpe, Crombie, Hill & Darrah, 1945), also by other bitten substances. The expressed juices of potato, mangold and carrot each have an activity of 6 for fed wireworms. A more detailed examination was made of potato juice. Potatoes were ground and the juice pressed out, spun to remove starch grains and filtered through kieselguhr. The activity of this juice was 6. The sugars in the juice, found to be 1%, would only account for an activity of 0–1 on a logarithmic scale, so that some other active substance or substances must have been present. After boiling and filtering,

followed by boiling with 2·5% of charcoal, a colourless protein-free solution of activity 6 was obtained. The addition of two volumes of alcohol gave a copious inactive precipitate, and a third volume precipitated colourless rhombic active crystals. These proved to be asparagine, the identification of which was confirmed by analysis of the copper salt. ($Cu(C_4H_7O_3N_2)_2$ requires Cu 19·6%, N 17·2%; found Cu 19·2%, N 17·0%.) Asparagine has an activity of 9–11 and glutamine the same activity. The distribution of asparagine and glutamine in potatoes has been examined by Neuberger & Sanger (1942). The means of their results in six varieties are respectively 0·26 and 0·21% of the fresh weight. This range would account for our observed activity of the juice, whether the activities of asparagine and glutamine are additive or not.

The substances causing orientation are shown in Table 4. All the activities shown in this table have been determined with wireworms starved for 2 days using

Table 4. *Substances eliciting orientation*

Asparagine	9	Glutamine	9
Aspartic acid	11	Glutamic acid	9
Malic acid	9	Citric acid	5
Succinic acid	9		
Fumaric acid	None		
Propionamide	9	Propionic acid	None
Acetamide	9	Acetic acid	None
Urea	9	Ammonia	None
Na acetate	None	Acetic acid	None
Na succinate	9	Succinic acid	9
Na citrate	5	Citric acid	5
Sucrose	2	Peptone	2
Glucose	2	Triolein	2
Fructose	2	Tannin	2

Inactive: acetic acid, alanine, ammonia, ascorbic acid, fumaric acid, glutaric acid, glycine, lactic acid, malonic acid, propionic acid, saccharin, sodium acetate, sodium chloride.

The figures show the activity as defined in the text. Since substances cannot be tested at concentrations greater than 1% activities of less than 2 are designated 'none'.

sand in the choice chamber. Activities were measured to the nearest 2 log units, i.e. an activity of 9 means that activity was greater than 9 but less than 11. Compounds which are not active at a concentration of 1% are considered to be completely inactive for practical purposes, and in the table their activity is specified as 'none' instead of <2. Substances causing orientation consist of a group of sugars all of activity 2–3 and peptone, triolein and tannic acid each of activity 2–3, and of a group of acids and amides with higher activities. In the latter group are several dibasic acids and amines widely distributed in plants. The amides of the lower fatty acids are active, but the acids themselves and ammonia are not. It is interesting that amides are the active compounds in buffalo dung which attract the Buffalo Fly *Lyperosia exigua*. (Krijgsman and Windred, 1933).

That *p*H has no effect between 4 and 8 has been shown by diluting the solution to be tested with inactive buffer solutions. No difference has been found between acids and their sodium salts. At such low concentrations as are used both the acid and the

salt are so nearly completely ionized that the anion must be the active agent. At a concentration of 1 in 10^8 acetic acid is about 99% dissociated and has a *p*H close to 7.

Glucose, sucrose, peptone, triolein, asparagine, aspartic acid and acetamide were tested with *A. sputator* as well as *A. lineatus-obscurus* with which all the substances were tested. No difference in behaviour was observed between the different species of wireworms. Nor were activities different at 16 and 23° C.

The tests described above were all performed in sand. In soil very different conditions obtain. The compounds used may be destroyed by micro-organisms or other means, and may also be adsorbed by the soil particles. Several substances have already been tested in air-dried soil. Further sterilization made no apparent difference to the rate of disappearance of the compounds tested. Table 5 shows the activities obtained.

Table 5. *Orientation activities in sand and in soil*

	Sand	Soil
Aspartic acid	11	3 or 4
Asparagine	9	3 or 4
Acetamide	9	4
Urea	9	1 or 2
Sucrose	2	2

The effect of starvation on the threshold of orientation of 10 individual wireworms (*A. sputator*) which were known to be active was tested in the plate apparatus (in sand). A response to the boundary between water and the solution in question was taken as indicating activity of the latter (§ IV (iv)). For all 10 wireworms when fed the activity of glucose was 2·1 but not 2·3, after 2 days' starvation 2·3 but not 2·5, and after 4 days' starvation 2·5 but not 2·7. After 7 days' starvation the activity of glucose was 2·7 but not 2·9 for 9 wireworms, and 2·9 but not 3·1 for the remaining one. No further change occurred with 14 days' starvation. The first track in glucose in Text-fig. 10*b* is made by the last-mentioned wireworm, the other two tracks by wireworms for whom the activity of glucose was 2·7 after 7 days' starvation. The orientation activity of glucose for wireworms thus increases with starvation parallel with biting activity if the latter be measured by the lowest concentration which causes any more biting than the controls (Text-fig. 7). For all 10 wireworms when fed the activity of asparagine was 9 but not 11. No change occurred with 2 days' starvation, but after 4 days activity was 11 but not 13 for all. After 7 days activity remained at 11 for 7 wireworms but went to 13 but not 15 for 3, including the same wireworm for whom the activity of glucose went to 2·9. Of the tracks in asparagine shown in Text-fig. 10*b*, the first is in asparagine of concentration 10^{-11} and the last 2 that of concentration 10^{-13}. No further changes occurred with 14 days' starvation. The limits of activity reached may be taken as the limits of sensitivity of the receptors concerned for glucose and asparagine respectively. Sensitivity to glucose seems to have approximately the same value whether biting or orientation is being exhibited.

(iii) *Relationship between biting and orientation*

While sugars and other bitten substances cause both reactions, other active substances cause only orientation. The wireworms must therefore be able to distinguish between these two groups. The sensitivity of the receptors to orientation substances other than sugars and other bitten substances is comparable to that of the most sensitive olfactory receptors, while their sensitivity to the biting groups is similar to that found in organs of taste. Man is sometimes classed as 'microsmatic' yet Bach (see Moncrieff, 1944, p. 80) records that for the human nose synthetic musk, vanillin and skatol have activities of the order of 10 or 11. Truly 'macrosmatic' mammals do not appear to have been subjected to critical olfactometer tests and such experiments would not be easy, but the following are the activities of some of the most active substances stimulating the olfactory receptors in some other animals: ethyl butyrate for the newt (*Triton cristatus*) in air and water 5·4 (Gertz, 1938), bromostyrene for the antenna of the honey bee (*Apis melliphora*) 3·3 (von Frisch, 1919), and skatol for the water beetle (*Hydrophilus piceus*) in water 6·2 (Ritter, 1936). Bromostyrene has an activity of 4 for man and the newt, i.e. about equal to that for the bee (Gertz, 1938). It is probable that the latter is much more sensitive to other naturally occurring odours. The activities of sucrose for the organs of taste of these animals are as follows: for the human tongue 2·2 (Parker, 1922, p. 143), for the newt's mouth 1·7 (Giersberg, 1926), for the honey bee's mouthparts 1·7 (von Frisch, 1934), and for the palps of *Hydrophilus piceus* 2·5 (Bauer, 1938). The wireworm receptors which respond to orientation substances may be presumed to facilitate the discovery of 'distant' sources of emanation, and it is interesting to note that these respond not to odours but, like those of aquatic animals, to dissolved substances at very low concentrations.

At first sight it may seem that orientation in substances which elicit biting may be caused merely by the wireworms stopping to bite, and that they elicit not two but only one response. But, as shown above, at certain times of year wireworms do not orientate to substances which normally elicit this response (Text-fig. 2). During such periods it was discovered that while wireworms would orient neither to sucrose nor to potato juice, they exhibited the full biting response to these substances. The average number of bites per 20 wireworms in 24 hr. was 582 (six experiments) on potato juice and 400 (three experiments) on 5% sucrose. Individual wireworms have also been observed to give the orientating response at a boundary of glucose in the plate apparatus, while not biting glucose on filter papers. Biting and orientation must therefore be distinct, and the response exhibited depends upon the physiological state of the animal. Wireworms may at different times exhibit both responses, bite without orientating, orientate without biting, or exhibit neither response.

(iv) *Orienting mechanism*

The mechanism of orientation of active wireworms to asparagine, glucose, sucrose and peptone was examined in the plate apparatus with soil as the medium. The

wireworms had been starved for 2 days. To see whether the speed of movement was less when the sense organs were being stimulated by an active substance, the length of track made in 1 hr. by the same wireworm (*A. obscurus-lineatus*) was measured first in water then in the substance. Table 6 shows that the average lengths of tracks made in an hour in each of the substances tested is less than that in water. If it be assumed that track length is proportional to speed of movement, it follows that wireworms exhibit orthokinesis to each of these substances (Fraenkel & Gunn, 1940). Orthokinesis forms an integral part of the responses of wireworms to humidity (Lees, 1943*a*) and temperature differences (Falconer, 1945*a*).

Table 6. *Relative distances travelled in 1 hr. by individual active wireworms in sand impregnated with water and various active substances*

Substance	No. exps.	Average distance (cm.) travelled per hour in		Ratio of velocity in substance to velocity in water
		Water	Substance	
Asparagine (0·2 %)	3	5·6	3·5	0·62
Glucose (2 %)	6	8·4	3·2	0·38
Triolein (2 %)	3	4·5	1·6	0·36
Peptone (2 %)	6	3·9	1·7	0·44

In all the experiments each individual moved slower in the substance than in water, and in a different test responded to a boundary.

In the choice chamber experiments a reduction of velocity on the bait side will lead to collection on that side. The situation is analogous to a vessel of gas divided by a non-conduction membrane with the separate masses of gas at different temperatures. For the pressure on each side of the membrane to be equal, corresponding to an equilibrium in the choice chamber in which an equal number of wireworms cross the boundary in opposite directions in unit time, the number of molecules in unit volume on the colder side must be greater. Without knowledge of the distribution of velocities in a wireworm population it is impossible to calculate the average retardation which will cause a given percentage to collect, but by making certain assumptions an approximation to the reduction in velocity can be found.

It is assumed that the velocity of all the wireworms on the water side is v and on the bait side w; that the change in velocity occurs immediately the dividing line is crossed, and that in the equilibrium state there are a wireworms on the water side and b on the bait side.

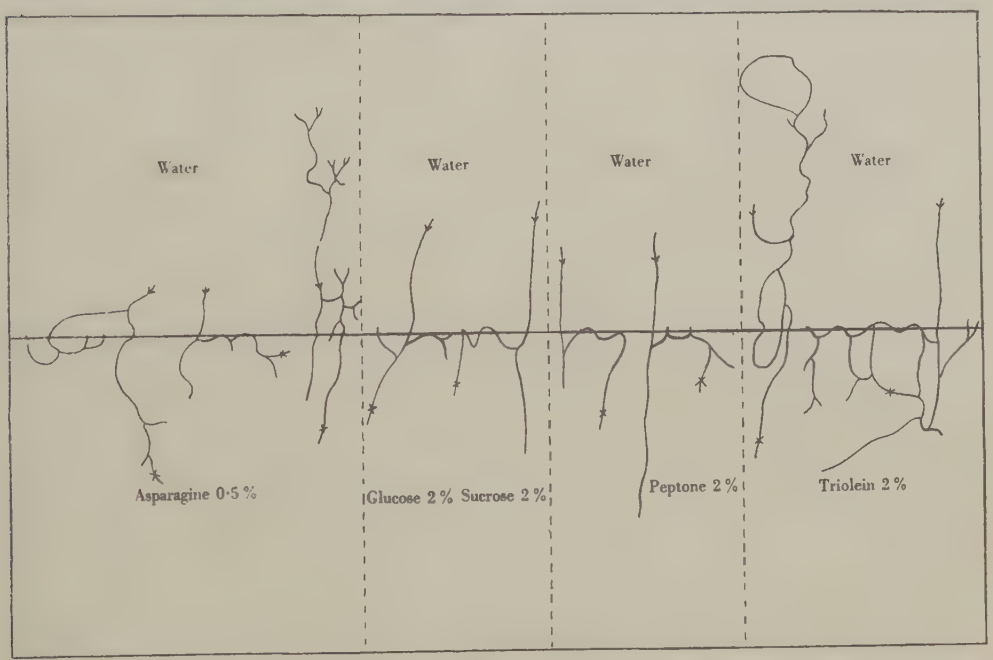
The number of wireworms crossing from water to bait in unit time will be proportional to av, where the constant of proportionality is dependent only on the dimensions of the water compartment. The number crossing from bait to water will be proportional to bw. Since the compartments are of equal size, at equilibrium $kav = kbw$, hence

$$1 + \frac{w}{v} = \frac{a+b}{b}.$$

In the analysis of results in the choice chamber the mean value for $\frac{b}{(a+b)}$, the fraction of wireworms on the bait side, is 0·64

$$\therefore 1+\frac{w}{v}=1{\cdot}56.$$

So the degree of orthokinesis, w/v, required to give a collection of 64% under these simplified conditions is 0·56. This shows fair agreement with the degree of orthokinesis measured in plate experiments, but since the wireworms used in the latter experiments had been specially selected and the degree of variation in such experiments is great, the other reactions which have been shown to occur at the

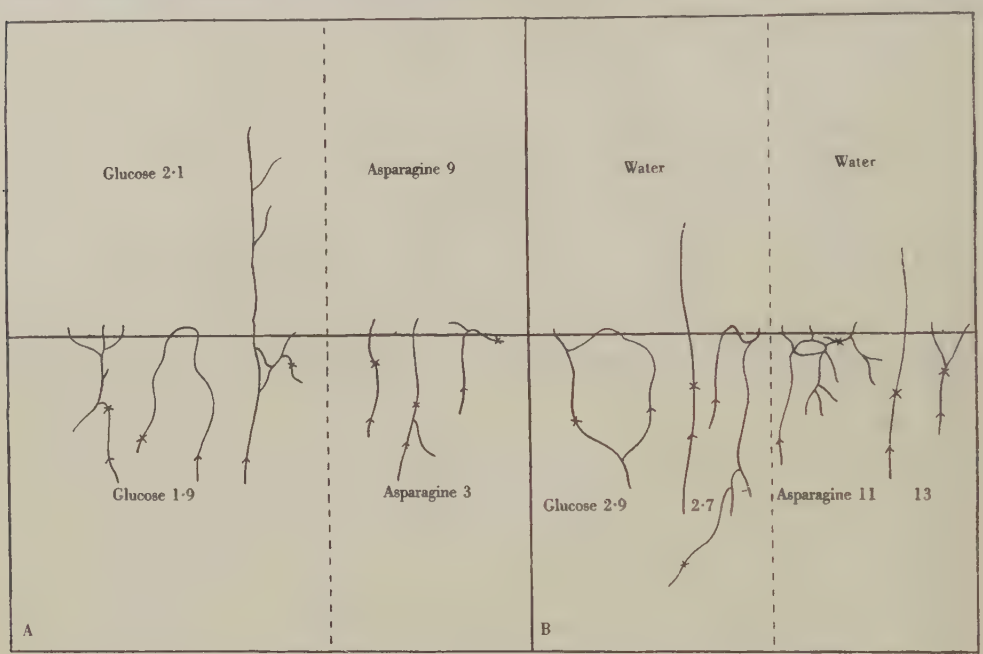


Text-fig. 9. Tracks made by wireworms responding at the boundary between water and various active substances in soil in the plate apparatus. The first track in each substance is by *A. sputator*, the others by *A. obscurus-lineatus*. An arrow indicates the point and direction in which individuals started, × where they were found at the end of the experiment.

boundary are not excluded by this calculation from playing an important part in causing collection on the bait side of the choice chamber.

To examine the possibility of other orientating mechanisms a boundary between water and solution was arranged in the soil in the plate apparatus. Wireworms were placed pointing towards the boundary sometimes on one side and sometimes on the other. They were then left in the dark and when they had wandered about for a suitable time (usually 2 hr.) the tracks were traced. Some individuals were indifferent to the boundary. Others responded as described below, examples of tracks made by those which, having started on the water side and crossed to the bait side, turned again and responded at the boundary, being shown in Text-fig. 9. The

movements of wireworms were also observed continuously in dim light. The usual behaviour was as follows. When proceeding from water into an active substance no response occurred at the boundary. But when proceeding from an active substance into water, the wireworms either backed along their tracks sometimes quite long distances and then sometimes made a side turning; or else proceeding forwards they executed a turn which brought them back into the active substance. Both forms of behaviour are to be seen in the tracks shown in Text-fig. 9. Sometimes wireworms moving out of the bait did not respond immediately at the boundary but went quite long distances into the water side before returning (cf. Text-fig. 10). The most reliable criterion for activity is therefore whether the wireworms are found on



Text-fig. 10. A. Tracks made by wireworms responding at the boundary between different concentrations (expressed as activities) of glucose and of asparagine respectively in sand. B. Tracks made by wireworms (starved for 7 days) in sand at a boundary between water and the lowest concentrations (expressed as activities) of glucose and asparagine respectively to which they respond. An arrow indicates the point and direction at which individuals started, × where they were found at the end of the experiment.

the bait side after two hours. This occurred every time with active wireworms and active substances. The responses to asparagine, glucose, sucrose, peptone and triolein all have the same character.

Lees (1943*a*) and Falconer (1945*a*) describe similar behaviour of wireworms at the boundary between two different humidities and temperatures, respectively. Both authors find difficulty in placing the behaviour exhibited in any of the categories differentiated by Fraenkel & Gunn (1940). Both argue against klinokinesis, for there is no evidence of increased turning on crossing the boundary from the

preferred side, and Lees (1943*a*) is further impressed by the supposition that turning would be impossible in nature since the animal is restricted by the limits of the burrow. He inclines to the view that the mechanism involved is klinotaxis, a directed movement involving the 'comparison' in successive intervals of time of the intensity of the stimulus to which the animal is reacting. But the forward turn sometimes executed when the wireworm is crossing the boundary from water into an active substance (and there is evidence for the same movement in the tracks published by Lees (1943*a*) and Falconer (1945*a*)) may be regarded as an example of an increased rate of change of direction, and also involves the construction of a new burrow. On the other hand, the reversing movement along the burrow back from the boundary is probably best regarded as klinotaxis. The facts do not permit a decision in favour of either klinotaxis or klinokinesis, and it is perhaps wisest to refrain from forcing the behaviour observed into either category (cf. Gunn & Walshe, 1942). It must be remembered that these categories were devised for the description of the behaviour of animals in media which do not restrict lateral movement, and that they represent a limitation imposed by the animal's own structure and sense organs and by the nature of the environmental situation.

Wireworms exhibiting orthokinesis responded to the boundary, and vice versa. Some wireworms were found to exhibit neither response. The responses of *A. sputator* and *A. obscurus-lineatus* are similar. In Text-fig. 9 the first track drawn for each substance is made by a *sputator* and the others by an *obscurus-lineatus* individual. Responses in soil and sand are also similar (cf. Text-figs. 9, 10).

Wireworms respond to a gradient of concentration of both glucose and asparagine. A boundary was arranged in sand in the plate apparatus between solutions of different concentrations. Active individuals (*A. obscurus-lineatus*) known to be responding to the weaker solution were placed in the stronger solution pointing towards the boundary. A response occurred with various differences in concentration. The tracks made when the gradient was between 1·26% (activity 1·9) and 0·8% (activity 2·1) glucose solutions, and between asparagine solutions of concentrations 10^{-9} and 10^{-3} respectively, are shown in Text-fig. 10 A.

V. FIELD EXPERIMENTS

Field experiments were carried out for two purposes: to discover (i) the effectiveness in the field of baits containing active substances, and (ii) the extent to which wireworms move about under natural conditions.

(i) *Effectiveness of baits in the field*

The baits consisted of blocks of paper pulp roughly $6 \times 3 \times 3$ cm. soaked in solutions or emulsions of an active substance. Blocks soaked in water were used as controls. Slices of potato were also tested as baits. The baits were distributed at random, as recommended by Fisher & Yates (1943), in holes 10 cm. deep and 16 cm. apart. The numbers of each kind of bait and of controls were equal. They were dug up at intervals of 2, 3 or 4 days after burial and the wireworms on each counted and removed.

Preliminary experiments were conducted in a badly infested greenhouse on chalky medium-heavy loam at Fulbourn. Six baits containing 2% glucose and 0·1% asparagine caught 35 wireworms (30 *A. obscurus-lineatus*, five *athous*) and the six controls 1 *athous* in the first 2 days. After 4 days baits and controls had each caught 1 more *obscurus-lineatus*. Potato slices were then put down in place of the baits and after 2 days caught 11 *obscurus-lineatus* and 3 *athous* to the controls none. Wireworms were therefore still present in the soil after the bait had become ineffective, suggesting that it must have been destroyed.

Table 7. *The number of wireworms* (A. obscurus-lineatus) *caught after the intervals shown on baits containing various substances* (*Dry Drayton*)

Exp.	Substance	No. baits	Total no. wireworms caught on day no.								No. caught per bait per 2 days previous to day no.							
			2	3	4	6	7	8	9	12	2	3	4	6	7	8	9	12
1	Glucose 2% and asparagine 0·1%	10	29	—	19	18	—	3	—	—	2·9	—	1·9	1·8	—	0·3	—	—
	Water	10	11	—	7	4	—	7	—	—	1·1	—	0·7	0·4	—	0·7	—	—
2	Asparagine 0·1%	8	—	8	—	5	—	—	8	—	—	0·7	—	0·4	—	—	0·7	—
	Glucose 2%	8	—	42	—	17	—	—	8	—	—	3·5	—	1·5	—	—	0·7	—
	Asparagine 0·1% and glucose 2%	8	—	38	—	18	—	—	5	—	—	3·2	—	1·5	—	—	0·4	—
	Water	8	—	1	—	5	—	—	6	—	—	0·1	—	0·4	—	—	0·5	—
3	Asparagine 0·1%	8	—	—	10	11	—	—	—	5	—	—	0·6	1·4	—	—	—	0·2
	Glucose 2%	8	—	—	19	20	—	—	—	0	—	—	1·2	2·5	—	—	—	0
	Asparagine 0·1% and glucose 2%	8	—	—	20	23	—	—	—	4	—	—	1·3	3·0	—	—	—	0·2
	Potato slices	8	—	—	15	9	—	—	—	137	—	—	0·9	1·1	—	—	—	5·7
	Water	8	—	—	16	16	—	—	—	2	—	—	1·0	2·0	—	—	—	0·1
4	Glucose 2%	8	—	33	—	—	9	—	—	—	—	2·8	—	—	0·6	—	—	—
	Peptone 2%	8	—	7	—	—	4	—	—	—	—	0·5	—	—	0·3	—	—	—
	Triolein 2%	8	—	36	—	—	22	—	—	—	—	3·0	—	—	1·4	—	—	—
	Potato juice (protein free)	8	—	12	—	—	6	—	—	—	—	1·0	—	—	0·4	—	—	—
	Water	8	—	11	—	—	0	—	—	—	—	0·9	—	—	0	—	—	—
5	Glucose 1%	8	—	—	26	—	8	—	—	—	—	—	1·6	—	0·7	—	—	—
	Triolein 1%	8	—	—	15	—	13	—	—	—	—	—	0·9	—	1·1	—	—	—
	Tannin 4%	8	—	—	7	—	2	—	—	—	—	—	0·4	—	0·2	—	—	—
	Potato juice (crude)	8	—	—	11	—	7	—	—	—	—	—	0·7	—	0·6	—	—	—
	Potato juice (protein free, twice conc.)	8	—	—	20	—	7	—	—	—	—	—	1·3	—	0·6	—	—	—
	Water	8	—	—	3	—	5	—	—	—	—	—	0·2	—	0·4	—	—	—

A series of experiments were now carried out with baits of various substances in the ground at the same time. The experiments were performed during April and May 1945 in a market garden on heavy boulder clay near Hardwick which had a high population of wireworms. Glucose as well as the water controls was present in every combination of baits, so that all the other substances or mixtures of substances may be compared with this. The results are shown in Table 7. It is clear from the first three experiments that glucose and glucose + asparagine are equally effective and catch more than the controls, while asparagine catches no more than the controls.

In a further experiment it was shown that 5 % asparagine is also ineffective. Glucose and glucose + asparagine have ceased to catch more than the controls by about 8 days. Potato slices, on the other hand, continue to be effective beyond this period, and in fact remain so as long as they are alive (see below). They catch at a lower rate than glucose. Exp. 3 shows also that when wireworms are not removed they will continue to accumulate on potato baits. One difference between glucose and asparagine is that whereas the former elicits biting as well as orientation the latter elicits only orientation. The explanation of the ineffectiveness of asparagine may then be that although it causes orientation it provides no food, so wireworms will not remain in its presence. Exps. 4 and 5 show that triolein has about the same effectiveness as glucose but lasts longer. Peptone is ineffective in spite of causing both orientation and biting in the laboratory, and so is tannin. Potato juice is ineffective until it is twice concentrated. There are reasons for believing that the failure of these baits is due to soil action. Peptone and potato juice are both very favourable substrates for bacteria. This may explain why the threshold of glucose in potato juice on blocks in the field is four times the threshold of glucose alone. Tannin would be precipitated by calcium compounds present in the soil. The wireworms caught in Exp. 6 were tested in the laboratory and found to collect in 0·1 % asparagine, in four trials 149 (= 63 %) out of 236 collecting on the bait side of the choice chamber.

Two further experiments were performed during June and July 1945 in a field on heavy gault clay near Barton. In the first the effectiveness of water, 2 % glucose, 1 % triolein and 1 % triolein + 0·5 % asparagine, were compared. In five trials with eight blocks of each kind representing forty baits of each kind and forty controls, the total numbers caught in 2 days were: control 30, glucose 99, triolein 147, triolein and asparagine mixture 169, representing 0·75, 2·5, 3·7 and 4·2 wireworms respectively per bait in 2 days. Triolein may be slightly more effective than glucose, but there is no significant difference between the last two figures, suggesting that asparagine does not enhance the effectiveness of triolein. In the second experiment different concentrations of glucose were compared. In 2 days the controls and 0·5, 1, 2, 4 and 8 % glucose baits caught 8, 14, 13, 21, 16 and 19 wireworms respectively per eight baits, showing that the concentration of glucose has no effect during this period.

(ii) *Movements of wireworms in the field*

A preliminary experiment was carried out during March 1945 in the Fulbourn greenhouse in which lettuce were growing and which contained a high proportion of wireworms. Groups of twelve potato slices were buried in square patches 4·5 sq.ft. in area in different parts of the greenhouse. The patches were classified according to whether they contained well-grown lettuce, poor lettuce or no lettuce. The poor quality and absence of lettuce was suspected as being due to damage by wireworms. The baits were dug up at intervals of 2 or 3 days and the wireworms counted and removed. Table 8 shows that the wireworms were not uniformly distributed but that density of population is correlated with density of lettuce, and that the *athous*

Table 8

Patch (lettuce type)	No. patches	Total no. caught in 16 days	Average per patch	% *athous*
A (good)	7	299	43	28
B (poor)	3	60	20	28
C (absent)	2	16	8	13

were evenly spread amongst the other species (*obscurus-lineatus*). The 13% in C is not significant, since it is based on only 16 wireworms. There was no significant difference between the numbers found in (197) and between (178) the rows.

As the population was reduced the average rate of catching per patch (twelve baits) fell in A patches from 7·5 during the first 2 days to 2·1 during the last 2 days, in B patches from 5·7 to 0·3, and in C patches from 2 to 0. After 16 days the soil in four A patches and in four adjacent unbaited 'A' patches of the same area was dug out to the depth of 1 ft. and sieved. Twenty-six wireworms or 6·5 per patch were found in the baited 'A' patches and 17 wireworms or 4·1 per patch in the unbaited 'A' patches. Both sets of patches were now baited with twelve potato slices as before. During the next 8 days the four previously baited A patches yielded 20 wireworms or 2·5 per patch for 2 days and the four previously unbaited 'A' patches

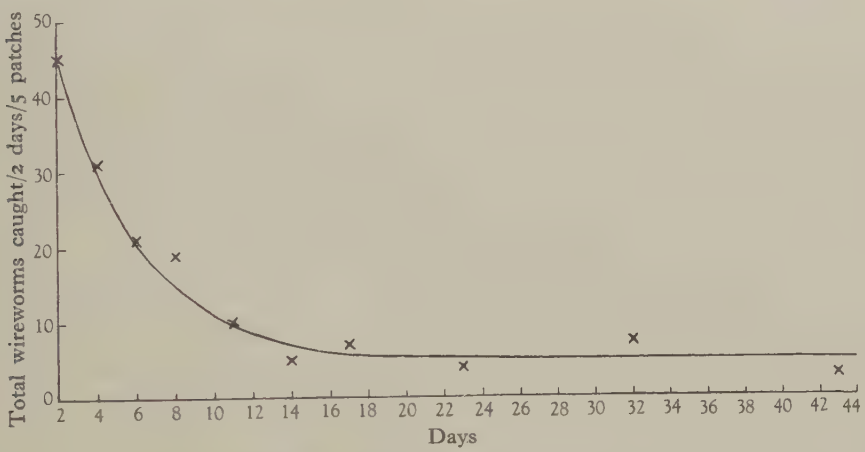
Table 9. *Number of wireworms caught after various intervals in patches 4·5 sq.ft. in area containing 12 potato slices per patch. A, control; B, bottoms sealed; C, sides sealed* (*Hardwick*) (*see text*)

Days	A	B	C
2	45	44	50
4	31	30	37
6	21	7	29
8	19	4	11
11	15	14	5
14	7	2	7
17	11	3	13
23	13	10	5
32	34	31	14
43	16	5	2
Average no. caught per 2 days for last 3 observations	5	3·5	1·5

29 wireworms or 3·6 per patch for 2 days. These must have wandered into the patches either from the side or from deeper down in the soil. The next experiment was designed on a more elaborate scale to investigate this point.

Fifteen square patches 4·5 sq.ft in area each containing potato slices were arranged in the market garden near Hardwick. The experiments were performed during April and May 1945. All vegetation was removed from the experimental area. The patches were of three kinds: A, control; B, with the bottom sealed off at a depth of 1 ft. by glass plates; and C, with the sides sealed to a depth of 1 ft. by glass plates.

The plates were inserted 2 days after the beginning of the experiment. The wireworms on the baits were counted and removed at intervals of a few days as in the previous experiment. Most of the potato slices remained alive during the whole period of the experiment; those which did not were replaced by fresh slices. Table 9 and Text-fig. 11 shows the total number of wireworms caught per patch in 2 days in A, B and C patches at successive points of time. The rate of catching decreases with time in all three, but faster in B and C than in A. The fact that the rate of catching falls rapidly and then continues at a steady level suggests that wireworms are wandering into the patches from outside. The smooth curve in Text-fig. 11 is calculated for the A patches on the assumptions that one-third of the wireworms in a patch are caught every 2 days, independent of population density, that the original density is 135 in five patches, that 6 wireworms wander into the patches in 2 days when the difference in rates of catching inside and outside the patches is 45 in 2 days, and that



Text-fig. 11. Rate of catching wireworms in five baited *A* patches (Table 9). The curve is calculated for the idealized scheme suggested in the text. The points show the actual rate of catching wireworms.

the number wandering in at any time is proportional to the difference in rates of catching inside and out. If these assumptions are true then the original population gradually decreases and the proportion among those caught of individuals which have wandered in increases until all those caught are such wanderers. A stable state is thus reached in which the rate of catching equals the rate of wandering. The average rate of catching for the last three records is 5 per 2 days, which means that the basic population should be 10. If the above assumptions are true the rate of wandering will be 5, i.e. all those caught are wanderers. It can be calculated in the same way that in all 92 wireworms have wandered into the patch. The total number caught is 212, and as shown above 10 remain. If a third of those present are caught the original population was 135, since 45 were caught in the first 2 days. The original population calculated from the above considerations about wandering would be $212+10-92=130$, which is a fairly close agreement. The total number caught in B is 150, the original population calculated as for A 132, and the number remaining 7,

therefore the number wandering in is $150+7-132=25$. The total number caught in C is 173, the original population 150, the number remaining 3, therefore the number wandering in is $173+3-150=26$. The glass partitions at sides and bottom thus both reduced the amount of wandering, from which it may be concluded that wandering occurs from all directions, and that there was a population of wireworms in the deeper layers of the soil which was being tapped during the course of the experiment.

An experiment was now performed during May and June 1945 on Mr W. Jackson's land on Swaffham Fen to observe directly the wandering of wireworms in the field. 500 wireworms were placed in a small hole 6 in. deep in a field known to be free of other wireworms. This was a fine light black fen soil, tightly packed below the top inch, which is an ideal medium for movement: all vegetation had been removed from the surface. Concentric circles were described round this point every foot up to 7 ft. from the centre, and the whole marked off into four quadrants. Rows of potato slices were placed along the circumferences of the concentric circles in alternate quadrants. These slices were examined at intervals and when, after 11 days, the outer ones began to catch wireworms the soil in the unbaited quadrants was dug out circle by circle and sieved to a depth of 6 in. then in part to a further 6 in. In the central hole 80 wireworms were found in the first 6 in. and 15 in the next 6 in. The numbers found in the unbaited quadrants of successive circles were 14, 10, 8, 11, 10 and 14 respectively, making a total of 67. The first circle of one quadrant was dug out to 1 ft. in depth, yielding 2 wireworms. The number found in this quadrant to 6 in. depth was 6. This suggests that about 20% ($\frac{17}{86}$) of the wireworms are below 6 in. Assuming that 20% of the wireworms were missed in sieving the total number in the two unbaited quadrants would then be $67+13$ (20% of 67 missed)$+16$ (20% of 78 below 6 in.)$=96$. The same number may be supposed to be present in the baited quadrants (actually 78 were caught on the baits so there may have been more in the baited quadrants). The number found in the centre was 95 plus say 10% missed making 105. The total number accounted for is therefore 295 out of 500. The shape of a graph of wireworm density (wireworms per sq.ft.) plotted against distance from the point of dispersal suggests that some of the wireworms unaccounted for are outside the 7 ft. zone, though some may have been destroyed by predators. This shows that wireworms can move over 7 ft. in 11 days, i.e. their average rate of migration in a given direction may be as much as 20 cm. per day.

Burrowing through the soil seems to be the usual method of movement of wireworms, but it is of interest to note that on several occasions when collecting in the field we found wireworms attacking the stems of wheat just above the surface of the soil. The position of the attacks showed that the wireworms had not penetrated up the interior of the stem from below, but had come to the surface and then begun the attack.

VI. DISCUSSION

It has been shown that plants in general contain substances giving two types of response. The orientation response is, in some cases, evoked by very low concentrations of the substance in solution. The biting response requires in general higher concentrations. The two responses were originally separated by finding larvae in a condition in which they showed the biting response only. The relation between sensitivity of the larvae to substances causing orientation and biting respectively, is quantitatively similar to the relation between the sensitivity in smell and taste in ourselves.

By examining a variety of substances it was found that the active chemical compounds in both cases were either related to or to be found among the compounds known to be present in the food plants.

When baits are used it is found that only the substances causing the biting response are effective in catching the wireworms. Further, the addition of an orientating substance to baits containing triolein does not increase the number caught. When triolein, which is insoluble in water, is compared in the field in dry soil as a bait with a solution of carbohydrate in water there is no significant difference in the numbers caught. This suggests strongly that the random type of burrowing is the main factor in such experimental conditions. Orthokinesis and the biting response together might thus account for all the results with artificial baits. This, however, would not exclude responses involving turning (klinotaxis and klinokinesis) playing a part in the finding of root systems under natural conditions.

The conception which it is necessary to define is that of 'random' burrowing. The first point that arises is that in field conditions the whole volume of the surface layer of the soil will not be equally explored by the wireworms. This is indicated as follows. The wireworms are considered to have a semi-permanent system of burrows. The growing plants themselves will, by the nature of the growth of their roots, produce a system of potential burrows or lines of least resistance. This is because of the sloughing off of the cortex of the roots some distance behind the growing point, and because plant rootlets are continuously being eliminated from the living plant. Further, plant roots will make use of the burrows left by the wireworms, and also of the potential burrows left by the dead roots of previous plants. Therefore there will be a definite connexion between the wandering through the soil of both the wireworms and the growing roots of plants.

It is readily seen how this can, independently of any further mechanism, increase the chance of larvae being found within the boundaries of the root system of the plants. It might partly explain the results of 'good cultivation' in diminishing the effect of the wireworm populations of the soil, and further show how vulnerable young plants may be in some cases where the roots actually pass into the semi-permanent system of burrows.

This would go far to account for the fact that in dealing with the chemical responses we can find no evidence of any effect due to percolation taking place

through an appreciable distance in the soil. The baits used in the experiments must be considered as intersecting a number of potential burrows or lines of least resistance. The plant-root system is, however, very different from the baits used.

It is just at this point that we could see a necessity for the two chemical responses. It is the general impression that the larvae rapidly find the main axis of the root system and the neighbourhood of the most actively growing part of the whole plant. The assumption is now made that, during the growth of the root system, as the root hairs are continuously eliminated some distance behind the growing root tips substances of the type of asparagine and aspartic acid will be set free into the soil. This will very slightly increase the target area of all the growing roots at the outside of the root system as a whole, and from the properties of the orientating response we could then infer that the presence of aspartic acid, etc., will tend to keep the larvae within the root system as a whole.

It must be emphasized that the wireworms in the soil have no reliable or precise general method of orientation. They can avoid extremes of temperature, and humidity below saturation, but they are in the dark and they are not sensitive to gravity. Thus one would expect chemical methods of orientation to be necessary. From the experiments described and by making use of the assumptions stated previously we can give a more detailed explanation of the method of food finding than was hitherto possible.

Finally, it may be concluded that the prospects of diverting an attack from the critical early stages of a crop are encouraging. The best time for this would presumably be before the wireworms have had the chance to establish burrow systems directly associated with the plants. The efficiency of any baiting method can, on the conclusions we have drawn from the work here described, readily be seen to depend greatly on the type and condition of the soil. To bring the bait method to its fullest practical efficiency in wireworm control the aim should be to produce a bait substance resistant to bacterial attack. This should then be mixed with a non-repellent contact poison. On these lines the method might have a wide application.

VII. SUMMARY

1. The behaviour of wireworms *Agriotes lineatus*, *obscurus* and *sputator* in relation to food and to chemical substances of plant origin has been investigated by various types of olfactometer and choice chamber as well as by field experiments. *A. lineatus* and *A. obscurus* cannot be distinguished in the larval stages but, when possible, results obtained with these two were checked with *A. sputator* and in no case was any significant difference found.

2. Although wireworms when seeking food will, under exceptional conditions, walk on the soil surface it has not been possible to detect any response to odours under such conditions.

3. When wireworms encounter certain plant juices or solutions containing either one or more of a number of carbohydrate, fatty or protein substances the biting response is elicited.

4. Of the carbohydrates all the sugars tested elicit biting, certain common plant sugars being among the most active in this respect. It appears that the polyhydric alcohol grouping is responsible for activity as in the case of the human sense of sweetness. The *p*H activity curve for biting (tested on glucose) shows a marked peak between 6 and 8—most plant juices having a *p*H of between 5 and 7.

5. Triolein is the only pure fat to which the wireworms have been found to give the biting response, but the sodium salts of certain fatty acids are active in this respect.

6. The proteins so far found to be active in eliciting biting are of animal origin while the plant proteins tested are inactive. Partially broken down proteins may be active though the parent proteins were not, but none of the amino-acids or mixtures of amino acids tested have proved active.

7. As with the feeding of a number of other insects, the threshold for biting is lowered progressively with starvation up to 7 days.

8. In a sand- (or soil-) filled chamber wireworms show the 'orientating response', as a result of which they tend to collect in that side of the chamber in which the sand is moistened with aqueous extracts of plant tissues and desert that side which is moistened with an equal quantity of water. This orientation can be induced by a number of the substances which cause the biting response as well as by aqueous solutions in very low concentration of several dibasic acids and amides (e.g. aspartic and asparagine acid) and related substances which are widely distributed in plant tissues. Thus while the activity of sucrose in causing orientation is 2–3 on a logarithmic scale that of aspartic acid is 11, the former sensitivity being comparable to that usually found in organs of taste, while the latter is of the order characteristic of olfactory responses.

9. The sensitivity for orientation tends to vary with the nutritional state (as well as with season and time in relation to moult) as it does for biting. After 7 days' starvation the sensitivity of some individual wireworms gives an activity of 3 for glucose and 13 for asparagine—figures which may be taken as indicating the limits of sensitivity of the receptors concerned. It is shown that orientating wireworms are sensitive to a gradient of active substances.

10. It is shown that orientation is partly the result of orthokinesis and partly of a type of behaviour which partakes both of klinokinesis and klinotaxis.

11. Field experiments are described in which artificial baits were employed. It is shown that burrowing, following the lines of least resistance in the soil but random in respect of chemical stimulation, is the primary basis of food finding. With paper-pulp blocks containing glucose or triolein as artificial baits random burrowing combined with orthokinesis and the biting reaction will cause a very considerable rate of catching. The extreme sensitivity to asparagine and related substances, while conferring no significant advantage when tested with a large rectangular paper-pulp bait, is regarded as a means of increasing the 'target area' of a root system and of retaining the insect in the vicinity of fine roots once they have been encountered—so serving as a fine adjustment in the food-finding process.

12. The prospects of developing the bait method are discussed. It is suggested

that combinations of a stable non-water-soluble bait substance with a non-repellent contact poison might provide a method of widespread application for protecting the critical early stages of crops, and that further research should be directed along these lines.

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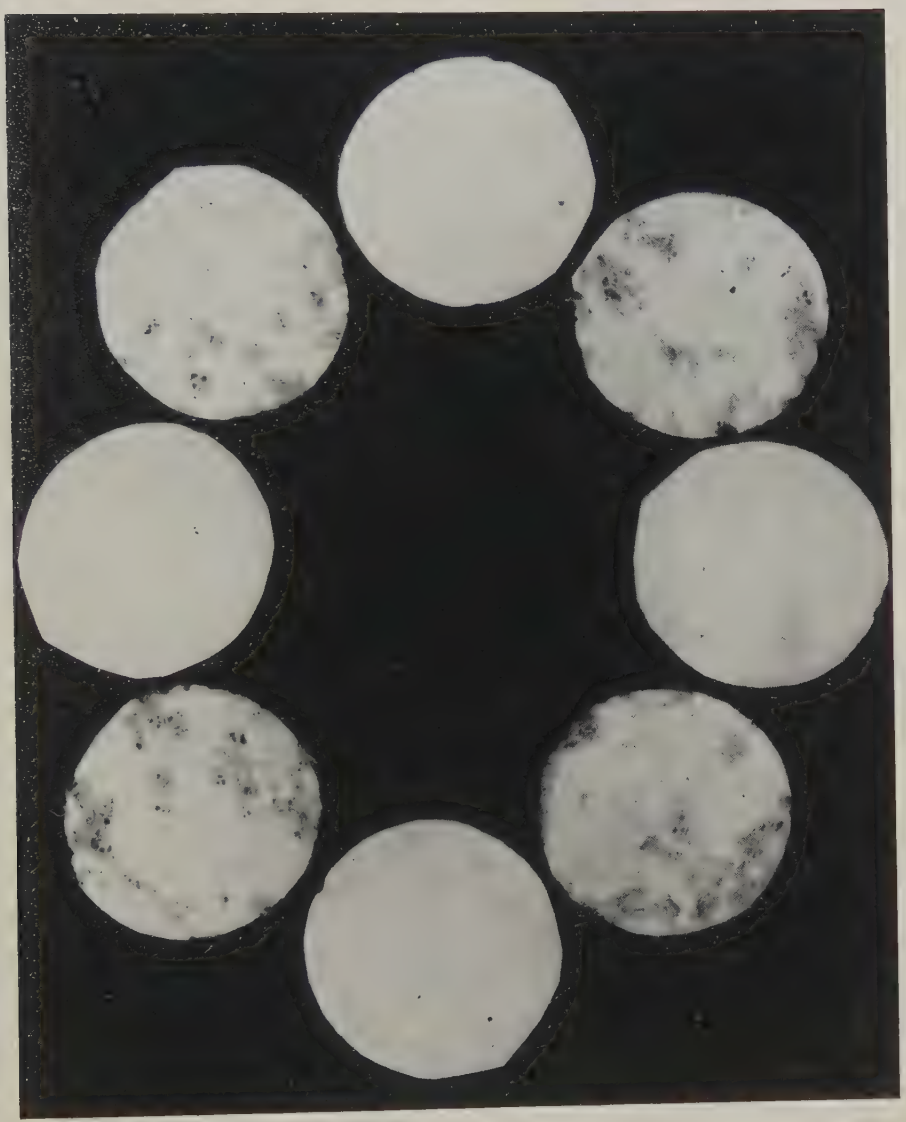
EXPLANATION OF PLATE 9

Photographs of filter papers bitten by wireworms. Those on the diagonals contain carrot juice, the others water (see text).



THORPE, CROMBIE *et. al.*—BEHAVIOUR OF WIREWORMS IN RESPONSE TO CHEMICAL STIMULATION

SPONTANEOUS RHYTHMICAL IMPEDANCE CHANGES IN THE EGG OF THE TROUT. II

By LORD ROTHSCHILD

(*Received* 20 *June* 1946)

(With Five Text-figures)

INTRODUCTION

Fertilized and unfertilized trout eggs exhibit periodic structural changes in their protoplasmic membranes after being in tap water for about 7 hr. The frequency of these changes is increased by an increase in temperature, while the changes themselves can be reversibly abolished by phenyl urethane (Hubbard & Rothschild, 1939: Rothschild, 1940). These periodic changes can best be measured electrically in terms of the impedance of the cell membrane, which is found to vary in an approximately sinusoidal manner, with a period of about 1·5 min. Measurement of impedance provides little information about membrane structure, as it involves both resistance and capacitance in living systems.* If, however, the observed impedance can be resolved into its resistive and capacitative components, information may be obtained about the structure and properties of the cell membrane. This has been done with great success in the case of *Nitella* and the squid nerve (Cole & Curtis, 1938, 1939), in which the propagated action potentials are associated with impedance variations which have been resolved into their resistive and capacitative components by an analytical method outlined later. It is found that the impedance variation is almost entirely resistive, the capacitance of the membrane remaining nearly, but not quite, constant. The fall in resistance during the propagation of an action potential provides an elegant electrical demonstration of the breakdown of membrane impermeability during activity. If, however, the impedance variation had been found to be mainly due to a change in capacitance, the whole picture of membrane reactivity during the passage of an action potential might have had to be modified.

In the case of the trout egg, there is no evidence of periodic changes in potential across the membrane, and it is therefore of special interest to resolve the periodic impedance changes into components which can be considered in terms of membrane structure. The experiments described in this paper were done just before the war and their analysis has therefore been delayed. They are in a sense preliminary, but the results are sufficiently definite to warrant publication.

MATERIAL

Fertilized and unfertilized eggs of *Salmo irideus* Gibbons and of *S. fario* were used. The egg is about 4·5 mm. in diameter. It resembles a plant cell in that the living part is restricted to a thin spherical shell, a few micra thick, within which there is a

* Cole (1941) interprets the electrical behaviour of nerve membranes as being due to inductance as well.

yolky globulin-containing solution whose electrical resistance varies between 100 and 200 ohm-cm. (Gray, 1920; Rothschild, 1946). Outside the living membrane, known as the vitelline membrane, there is a further membrane, the chorion, separated from the vitelline membrane by the perivitelline space. The chorion and perivitelline space are about 100 μ thick in normal conditions. As the chorion is freely permeable to water and small molecules, the perivitelline space has approximately the same composition as the external medium.

APPARATUS

The impedance of the vitelline membrane was measured by placing the egg in an electrolytic cell in the unknown arm of an alternating current bridge, and balancing this unknown arm at different frequencies by a resistance R_p in parallel with a capacitance C_p in the standard arm. The apparatus has been fully described elsewhere (Hubbard & Rothschild, 1939). In previous experiments the electrolytic cell was usually cubical, with platinized platinum electrodes forming two opposite faces of the cell. In these experiments, hollow hemispherical platinized platinum electrodes, whose separation could be adjusted, were used, close up to the egg surface. This procedure makes Maxwell's well-known equation involving the resistance of suspensions of spheres inapplicable. This only affects the quantitative measurements of membrane resistance and capacitance with which we are not directly concerned in this paper.

ANALYTICAL PROCEDURE

At any given frequency, the impedance of the egg in its electrolytic cell can be expressed in terms of the parallel resistance R_p and capacitance C_p in the standard arm of the a.c. bridge, provided that the impedance does not vary. If the values of R_p and C_p so obtained are converted into the equivalent series form, R_s and $-jX_s$, where R_s is the equivalent series resistance and $-jX_s$ is the equivalent series reactance [$X_s = 1/\omega C_s$, where $\omega = 2\pi . \nu$ (frequency)], the locus of the co-ordinates R_s, $-jX_s$ at various frequencies will be an arc of a semicircle, cutting the resistance axis at infinite and zero frequency. The phase angle of the capacitative part of the membrane will be half the angle formed by the radii from R_s at $\nu = \infty$ and R_s at $\nu = 0$. Each co-ordinate R_s, $-jX_s$ is the terminal of the impedance vector at the frequency in question, as $|Z| = \sqrt{(R_s^2 + X_s^2)}$, where $|Z|$ is the absolute magnitude of the impedance. A description of the 'Circle Diagram' analytical technique has been given in further detail by Cole (1928).

This analysis holds good for any combination of resistances and a single capacitative element, or as it is sometimes called when the phase angle of the capacitative element is constant and less than $\frac{1}{2}\pi$, a dielectric impedance element.

Suppose that a circle diagram has been obtained (Fig. 1 *a*), and that the cell membrane is treated in some way so that only its resistance falls, impedance measurements being made at times t_0, t_1, t_2, ..., during the change of resistance. Cole & Curtis (1938) showed that at each frequency the locus of the terminals of the impedance vectors corresponding to measurements at $t_0, t_1, t_2, \ldots$, will be part of

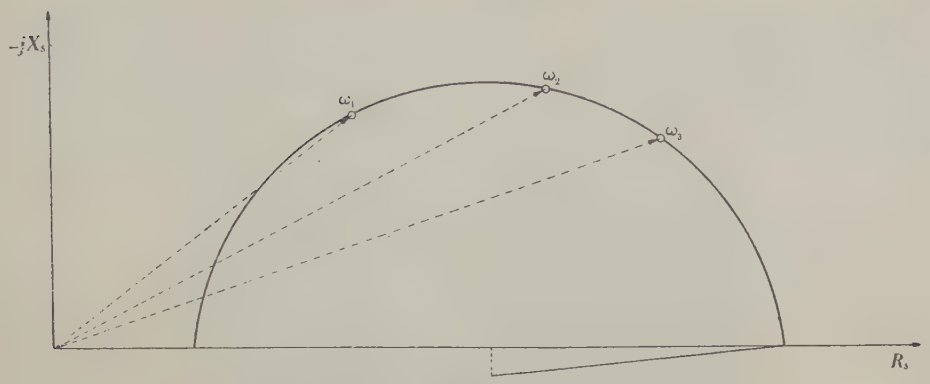


Fig. 1 *a*

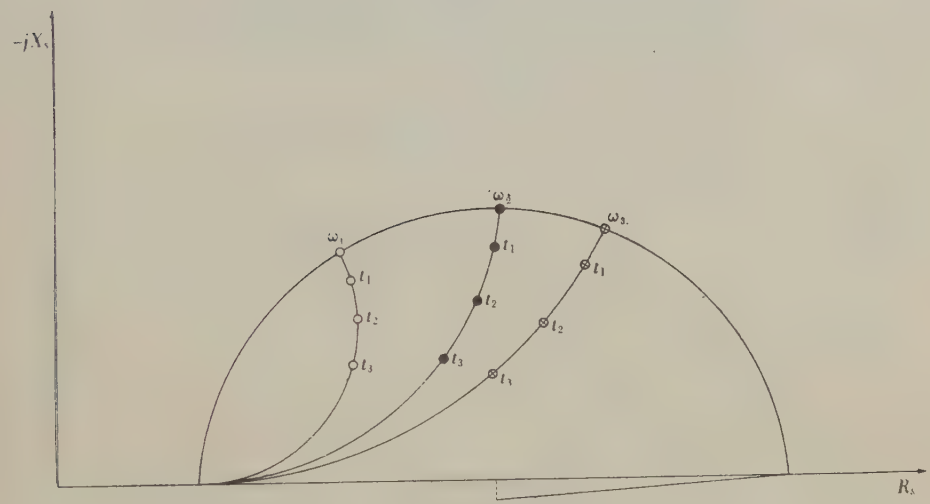


Fig. 1 *b*

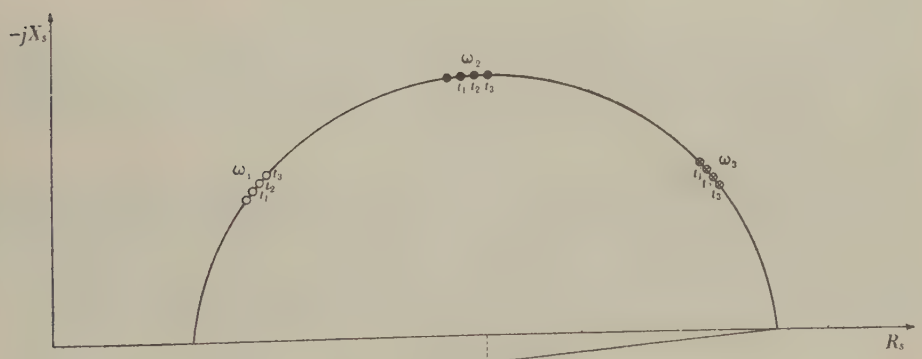


Fig. 1 *c*

Fig. 1. *a*, 'resting' circle diagram showing impedance vectors at three frequencies ω_1, ω_2 and ω_3. *b*, the effect of a membrane resistance variation with time on the terminals of the impedance vectors at frequencies ω_1, ω_2 and ω_3. *c*, the effect of a membrane capacitance variation with time on the terminals of the impedance vectors at frequencies ω_1, ω_2 and ω_3.

another semicircle, the circumference of which passes through the terminal of the t_0 impedance vector, and is tangent to the resistance axis at R_∞. The effect on the 'resting' circle diagram of a change with time in membrane resistance is shown in Fig. 1*b*.

If, however, the treatment of the membrane results only in a change of capacitance, the terminals of the impedance vectors at each frequency and at t_0, t_1, t_2, ... *remain* on the original arc of a circle but move round the perimeter towards R_0 when the capacitance decreases, and towards R_∞ when it increases.

Although the effect on the 'resting' circle diagram of a change in membrane resistance is easy to see in Fig. 1*b*, the effect of a change in capacitance is somewhat confusing because time and reactance are both plotted in the R_s, X_s plane. As a result, during a pure capacitance change with time, the terminal of the impedance vector at a particular frequency ν_j at time t_0 looks as if it becomes the terminal of another impedance vector at another frequency ν_i, but at the same time t_0. In reality

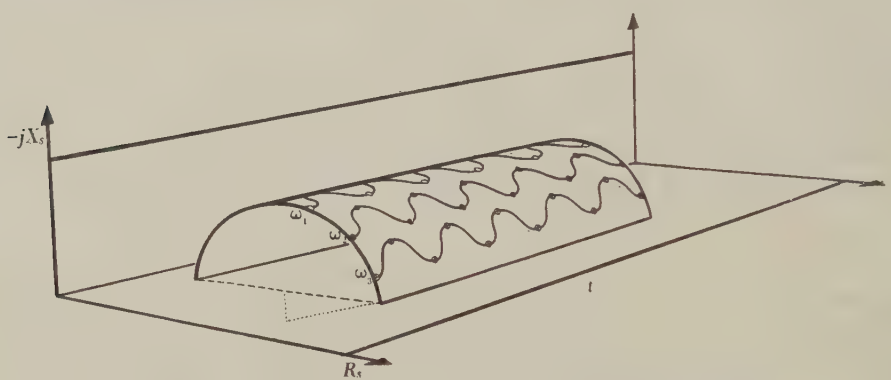


Fig. 2. A variation of membrane capacitance with time, plotted in three dimensions, R_s, X_s and t.

it is the *same* impedance vector, at the *same* frequency ν_j, but at a different time t_1. If, however, the capacitance change is plotted in three dimensions, R_s, X_s and t, the situation becomes clearer. A sinusoidal variation of capacitance with time is plotted in this way in Fig. 2.

If the change in the membrane is due to a change of resistance *and* capacitance, the terminals of the time impedance vectors will occupy positions intermediate between the two extremes. This was found in the case of *Nitella* (Cole & Curtis, 1938), in which the membrane capacitance changes by 15% and the membrane resistance by a factor of 200, during the passage of an action potential.

RESULTS

When the impedance of the vitelline membrane of a trout egg is changing rhythmically, the detector across the a.c. bridge varies as in Fig. 3*a*. The reason that the record is bilaterally symmetrical with respect to the time axis has been explained in a

previous paper (Hubbard & Rothschild, 1939).* The actual variation of impedance with time is shown in Fig. 3*b*, which is a tracing from the envelope curve in Fig. 3*a*. If instead of photographing the impedance change, the a.c. bridge is kept balanced by hand adjustment of the parallel resistance and capacitance in the bridge standard

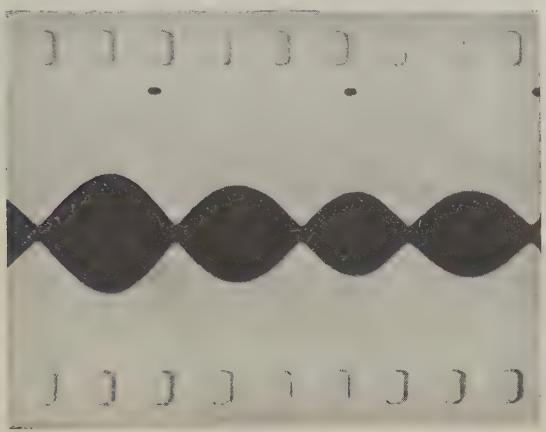


Fig. 3*a*

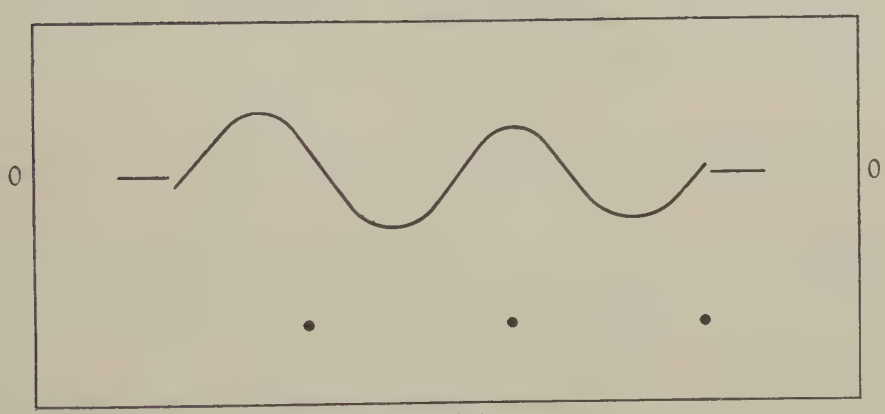


Fig. 3*b*

Fig. 3. *a*, periodic impedance change recorded oscillographically by detector across a.c. bridge. Time-marker, minutes. *b*, analysis of Fig. 3*a* showing variation of impedance with time. The horizontal line shows the balance point, at which the change in impedance $\Delta Z = 0$.

arm, a series of R_p and C_p values which repeat every 1·5 min.; will be obtained. This is shown in Fig. 4. The periodic variations in R_p and C_p can also be obtained by the 'ellipse' method of analysis (Cole & Curtis, 1938; Rothschild, 1940), but the frequency of the changes in the trout egg are sufficiently low to permit hand balancing.

* This can be explained briefly as follows. The oscilloscope measures the potential across the bridge. At balance this potential is zero; at off balance the potential oscillates around zero, because the source is alternating. Therefore in every cycle the potential is positive ('above' zero), and negative ('below' zero).

This procedure is repeated at a number of frequencies, and it is obvious from the section entitled 'Analytical procedure' that if the change in membrane impedance is due to a change in resistance, a series of impedance vector terminals, in the positions shown in Fig. 1*b*, will be obtained; while if the change is capacitative, the terminals will be as in Figs. 1*c* or 2; and if the change is due to a change in resistance *and* capacitance, the terminals will occupy positions intermediate between these two extremes. As the trout egg undergoes spontaneous changes in impedance, it cannot, as it were, be kept quiet while a static circle diagram is made, and then have its impedance changed for comparison of the resistive and capacitative parameters with the resting condition. Consequently, there is no 'resting circle' diagram for the trout egg, but nevertheless it is easy to see whether the maximum and minimum values of R_p and C_p, converted into the equivalent R_s, $-jX_s$ form, are in the

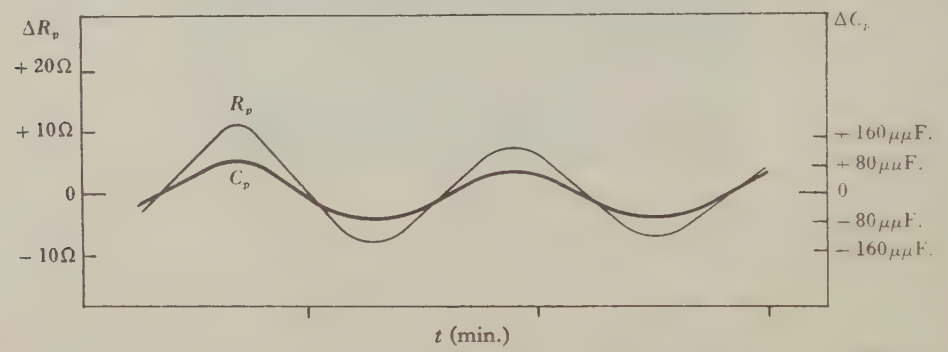


Fig. 4. Variation of parallel resistance R_p and C_p in standard arm of a.c. bridge during trout egg impedance change in unknown arm.

capacitance-change or the resistance-change positions. Two actual runs at various frequencies are shown in Fig. 5,* from which it is clearly seen that the impedance change is almost entirely capacitative. Three eggs were examined in this way over the whole frequency spectrum, which for this system is 0·2–50 kc. It is, however, also possible to tell whether the change is capacitative or resistive when the effect is examined at only one frequency, and particularly if the frequency in question is not too far in either direction from the 'characteristic frequency', at which $-jX_s$ is a maximum. Near this frequency, a resistance change in the membrane will cause the terminal of the impedance vector to oscillate almost vertically with time, while a capacitance change will cause an almost horizontal oscillation with time. Ten eggs have been examined in this way and all show the same result: that the rhythmical impedance change is almost entirely capacitative.

* Prof. K. S. Cole has drawn my attention to the fact that if the membrane impedance can be expressed in the form $Z_3 = K\omega^{-\alpha}$, where K is a constant, and $\log Z_3/K$ is plotted against $\log \omega$, there is a discrepancy between the slope of this line $(-\alpha)$ and the phase angle across the capacitance of the membrane as measured in the circle diagram $(\phi = \frac{1}{2}\pi\alpha)$. This discrepancy, which only occurs significantly in the first of the circle diagrams in Fig. 5, may be due to trout eggs not always obeying the empirical $Z_3 = K\omega^{-\alpha}$ rule; on the other hand, this particular batch of eggs had unusually variable phase angles. Whether this was due to an inherent variability in the eggs or to an extraneous factor is not known.

For reasons explained in the Introduction, the absolute value of the membrane capacitance per unit area cannot be determined in these experiments, though from other experiments it is known to be about 0·5 μF.cm.$^{-2}$ at 1 kc. (Rothschild, 1946). The percentage change in capacitance, due to the rhythmical change in the

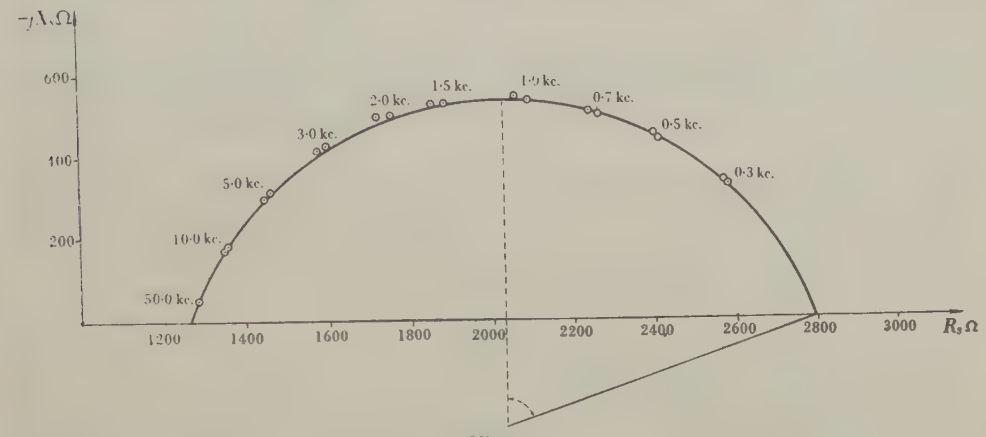


Fig. 5 *a*

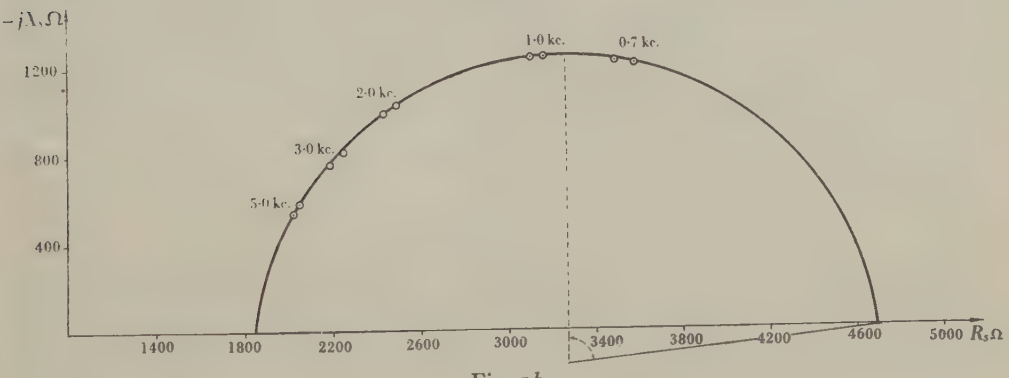


Fig. 5 *b*

Fig. 5. Circle diagrams showing periodic capacitance change in two trout eggs at various frequencies. The distance between each pair of circles represents one-half cycle, i.e. about 0·8 min.

membrane structure, can be roughly calculated. The equation for the capacitance of a bi-phase sphere in an aqueous medium is

$$c_{3i} = \frac{1}{a\omega_i} \frac{2(r_0 - r_\infty)}{(2 + r_1/r_0)(1 - r_1/r_0) r_0^2 \sin\phi} \left(\frac{\omega_i}{\omega}\right)^{\alpha}, \qquad (1)$$

where c_{3i} = capacitance in μF.cm.$^{-2}$ at a frequency $\nu_i = i$ cyc. sec.$^{-1}$,

a = radius in cm.,

$r_0 = R_s/k$ at $\nu = 0$,

k = electrolytic cell constant,

$r_\infty = R_s/k$ at $\nu = \infty$,

r_1 = specific resistance of external medium,

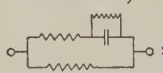
$\omega_i = 2\pi \times \nu_i$, where $\nu_i = i$ cyc. sec.$^{-1}$,
$\bar{\omega} = 2\pi \times \bar{\nu}$, where $\bar{\nu}$ = frequency at which X_s is a maximum,
ϕ = phase angle between current and voltage across the capacitative part of the cell membrane,
and $\alpha = 2\phi/\pi$.

This equation involves the assumption that the membrane resistance is infinite and that the membrane impedance can be expressed in the form $Z_3 = K\omega^{-\alpha}$, where K is a constant. The validity of these assumptions does not affect the experiments described in this paper.*

As no change in volume has been observed during the impedance cycle, and as there is no indication of any membrane resistance change, a, ω_i, r_0, r_∞, r_1, ϕ, and ω_i^α in the above equation are constant, the only variable during the impedance cycle being $\bar{\omega}$. The equation can therefore be written

$$c_{3i} = K\bar{\omega}^{-\alpha}, \tag{2}$$

where ω and α can be measured in the circle diagram.

From this equation and measurements in Fig. 5, the maximum percentage change in capacitance per cycle is found to be between 5 and 10% at about 1 kc. This may be compared with the percentage changes in R_p and C_p in the a.c. bridge standard arm which are 0·4 and 4·0 respectively. It is hardly necessary to mention that a change in resistance in the bridge standard arm in no way implies a resistance change in the unknown arm, because the equivalent circuit in the unknown arm can be represented as  , while that in the standard arm is  .

DISCUSSION

Capacitance is considered to be a characteristic of the cell membrane which remains constant under widely different conditions, and the periodic capacitance changes that occur in the trout egg are therefore of special interest. In discussing the membrane capacitance, Cole (1940) says: 'The singularly small changes of this ion impermeable part of the cell membrane in injury, death, current flow and excitation—where the ion permeability may change ten or a thousand fold—leads us to picture the ion impermeable structure as a massive, inert and durable framework, occupying almost the entire bulk of the membrane, with the ion permeability represented by at most a small percentage of the membrane volume.

'In contrast to the ion impermeability, the ion permeability as measured electrically has considerable functional significance and its changes reflect—or perhaps cause—a variety of physiological and pathological phenomena.'

The number of different types of cells whose plasma membranes have been electrically examined at all is small, and within this group there are few whose membranes can be made to alter their properties, and even fewer whose membranes

* It also involves the assumption that Maxwell's equation, referred to earlier on, is applicable. This is not the case because of the shape of the electrodes and their proximity to the egg, but the equation can be used to get a rough idea of the order of magnitude of the change.

do so spontaneously. In those that have been examined, membrane reactivity is almost entirely associated with changes in resistance rather than capacitance, though as mentioned earlier, there is a 15% change in membrane capacitance during the propagation of an action potential in *Nitella*; in the squid axon, the decrease in membrane capacitance during activity is only 2% (Cole & Curtis, 1939). The relationship between resistance and capacitance changes in active muscle membranes may well be of the same order. The only other cases in which changes in membrane structure are associated with changes of capacitance are in the eggs of *Hipponoe esculentus* and *Arbacia punctulata*, in which there is an increase of over 100% in capacitance after fertilization (Cole, 1938). In other eggs, however, fertilization has no effect on membrane capacitance (Cole & Guttman, 1942; Rothschild, 1946).

The question arises as to what functional importance is to be attached to the capacitance changes in *Nitella* and the squid axon during activity, and similarly what importance is to be attached to the spontaneous capacitance changes in the trout egg. The relatively great size of the resistance change during activity in the former is not necessarily a reason for ignoring the changes in capacitance or considering them as of little functional interest. The recent work of Hodgkin & Huxley (1945) on the variation of potential across the squid axon membrane during activity, where the potential not only decreases to zero but actually reverses in sign to the extent of 15 mV., shows that the 'uncorking' picture of the membrane during activity, with release of potassium into the external medium, may not be adequate. In the present state of our knowledge of the cell membrane it is difficult to say what effect a change in an oriented layer on the cell surface, causing a change in potential of 15 mV., would have on the capacitance. Conversely it is difficult to prophesy what effect a change of 5% in capacitance would have on the potential.

In all cells so far examined, with the exception of the trout egg, a change in impedance has been found to be associated with a decrease in resistance and potential. The position is not the same in the trout's egg because there is no evidence of periodic potential changes across the vitelline membrane, though it is possible that the insertion of a micro-electrode into the inside of the egg, which is involved in such measurements (Pumphrey, 1931), might interfere with such changes. Apart from this, the capacitance change is small and potential changes associated with it might have been too small to notice, particularly as the experiments were done with a quite different end in view.

If, however, one argues that a small change in capacitance may only be associated with a small change in potential, it is hardly consistent to argue at the same time that the significant reversal of potential during squid axon activity may only be associated with a diminutive change in capacitance.

Further experiments, particularly involving variations in the environment, may shed some light on the origin and significance of these spontaneous rhythmical changes in capacitance, both as regards their spontaneity and the fact that they are capacitative. Most cells which are capable of propagating action potentials exhibit spontaneous rhythmical potential changes under certain environmental conditions;

but as the impedance changes associated with their potential fluctuations will probably be found to be mainly resistive, they are unlikely to assist much in the interpretat on of the experiments recorded in this paper.

When the membrane resistance is very high, a relatively small change in it may be difficult to see in the circle diagram. Close inspection of Fig. 5 shows some very slight indications of a change in membrane resistance. The existence or otherwise of a change in membrane resistance during the impedance cycle could probably be determined more easily by d.c. than a.c. measurements.

SUMMARY

1. The spontaneous rhythmical impedance changes that occur in fertilized and unfertilized eggs of the rainbow trout after about 7 hr. immersion in tap water have been analysed. They are found to be almost entirely capacitative and correspond to a change of between 5 and 10% when measured at a frequency of about 1·0 kc. The possibility of any associated resistance change being virtually unobservable is discussed.

2. These changes, which have not so far been recorded in other living material, are compared with the impedance changes that occur in nerve cells and *Nitella*.

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AN ISOLATED NERVE-MUSCLE PREPARATION FROM *ASCARIS LUMBRICOIDES*

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(With Five Text-figures)

INTRODUCTION

In an earlier publication (Baldwin, 1943) we described a method for the detection and approximate measurement of anthelminthic potency. The method consisted essentially in recording by a kymographic technique the movements executed by short, sausage-like fragments of *Ascaris lumbricoides* (from the pig), prepared by tying off portions of the worm between tightly drawn silk ligatures. It was shown that there exists a high degree of correlation between anthelminthic potency in a given drug and positive reactions to it (i.e. eventual contracture or paralysis) on the part of our preparations. On the other hand, numerous drugs devoid of anthelminthic efficacy elicited no response, and among these were acetylcholine, adrenaline and a number of other sympathetico- and parasympathetico-mimetics.

In the past it has been almost standard practice in the chemotherapeutic assay of anthelminthic potency to carry out *in vitro* tests on tissues prepared from the body wall of earthworms and leeches (e.g. Trendelenburg, 1916; Lautenschläger, 1921; von Oettingen, 1929; Rosenmund & Schapiro, 1934; Oelkers & Rathje, 1941). These annelid materials are notoriously sensitive to the action of drugs of many different kinds. Earthworm muscle, taken from the body wall, responds to acetylcholine, eserine, choline, pilocarpine, to adrenaline, to ephedrine, and to cocaine, strychnine, caffeine and many others, none of which evoked any response from the *Ascaris* material used in our earlier experiments. The musculature of the alimentary tract of the earthworm likewise responds to many of these compounds (Wu, 1939). These facts suggest that either (*a*) the nematode cuticle, which was present in our preparations, is selectively permeable to some drugs and impermeable to others, or else (*b*) that the neuro-muscular apparatus of the nematode differs, perhaps fundamentally, from that of annelids. In either case the use of annelid material in the study of anthelminthics is not only illogical (cf. Lamson & Ward, 1936) but fundamentally unsound, and should be abandoned forthwith. In the hope of gaining more precise information we attempted to prepare from *Ascaris* functional fragments of the body-wall musculature of a kind in which there should be no cuticular barrier, so that a comparative study of the physiology and pharmacology of nematode material might be attempted.

The need for such a preparation has long been felt, not only for studies of anthelminthics, but also for the sake of the light that might be thrown with its aid upon

the neuro-muscular physiology of this phylum. Very little is known about the physiology of the Nematoda as a whole (see Lapage, 1937). Morphologically, as is well known, its members are very peculiar indeed and, from the biochemical point of view also, numerous peculiarities have been noted, such, for example, as the presence in the tissues of remarkably high concentrations of volatile fatty acids (see p. 284) and the occurrence of the apparently unique compound, ascaryl alcohol (Flury, 1912; Schulz & Becker, 1933). It is therefore to be anticipated that the physiology of the nematodes might also present some unusual features.

A few experiments on isolated *Ascaris* muscle were carried out by Trendelenburg (1916) in the course of his classical researches on the pharmacology of santonin and its derivatives, but the only record published in his paper does little to support his claim 'Das die Regenwürmer in derselben Weise wie die Spülwürmer auf Santonin mit Erregung reagieren würden, schien höchst wahrscheinlich'. Trendelenburg's authority appears to have been largely responsible for the introduction and subsequent wide employment of annelid tissues in anthelminthic studies: indeed, he himself soon abandoned nematode in favour of earthworm muscle because of the extreme difficulty of removing the cuticle of *Ascaris* from the muscular layer without damaging the latter. Our own experience has amply confirmed this difficulty.

We have now devised a simple preparation by which the musculature can be exposed directly to the action of drugs without previous removal of the cuticle. Observations on the physiology and pharmacology of preparations of this kind will be reported in further publications: for the present we propose to describe only the operative procedure, the general treatment and the normal behaviour of the new preparations.

MATERIAL AND METHODS

The collection of our animals, the general conditions of their maintenance in the laboratory and the experimental procedure were substantially the same as in our previous work (Baldwin, 1943). We have, however, adopted a new 'keeping medium'. We were fortunate in having at our disposal the results of a number of analyses, each obtained from a number of pooled samples, of the body fluid of *Ascaris* and of the (centrifuged) contents of the small intestine of pigs, and are very much indebted to Prof. A. D. Hobson, Dr W. Stephenson and Dr A. Eden for permission to make use of these data. The variations between one mixed sample and the next were considerable, and large enough, in our opinion, to justify the use of rounded average figures: these are set out in Table 1.

Table 1. *Composition of* Ascaris *body fluid, contents of small intestine of pig, and old 'keeping medium'*

	m*M* total					Equiv. m*M* NaCl	
	Na	K	Ca	Mg	Cl	Conduct.	Osm. press.
Ascaris, body fluid	130	25	6	5	53	143	198
Pig, gut contents	124	27	14	6	61	174	257
Old 'keeping medium' (Baldwin, 1943)	136	2·7	1·8	0·4	—	—	—

A striking feature of these new data is the great disparity between the amounts of chloride and total base. If the figures given by McCance (1936) for the human subject are in any way applicable to the pig, it is very probable that the deficit of anions in the pig-gut contents must be largely made up by bicarbonate. In view of the mutual replaceability of chloride and bicarbonate ions in most biological systems we carried out some survival experiments on intact worms in media made up from the chlorides Na, K, Ca and Mg in the proportions indicated by the analytical data for the pig-gut contents, but most of the specimens died within 2 or 3 days, i.e. much earlier than in the very dissimilar medium employed in our earlier work. This, we suspect, is largely due to the high [Ca^{++}] of the mixture; the data of Table 1 correspond to total and not to ionic concentrations.

Much better results were obtained with media patterned on the body fluid, buffered to *p*H 6·7 with phosphate and having a total salinity equivalent to 170 m*M* NaCl, a concentration very similar to that of the electrolytic components of the pig-gut contents (174 m*M*). In relative composition this saline closely resembles the natural external medium of the worm (see Table 3) except in so far as the Ca content and total osmotic pressure are concerned. Worms kept in this medium appeared to be more active than in our older 'keeping medium', individual specimens surviving as a rule for 4–12 days, but not longer than in the 'old keeping medium'. We adopted the newer medium on account of its closer resemblance to the natural environment of the parasites. Experience showed that useful results are only occasionally to be obtained with worms that have been in the laboratory for more than 24 hr., and we did not therefore go any further into the question of survival.

The new 'keeping medium' is prepared as follows. We keep a stock of concentrated medium containing 75·5 g. NaCl, 14·2 g. KCl, 13·1 g. $CaCl_2.6H_2O$ and 10·2 g. $MgCl_2.6H_2O$ per litre. A stock phosphate buffer is prepared containing 250 ml. 0·2 *M* KH_2PO_4 and 21 ml. *N* NaOH made up to 1 l. One volume of the concentrated stock receives 1 vol. of the stock buffer and is diluted to 10 vol. with distilled water and warmed to 38° C. for use. The product has the following properties:

	m*M*
Na^+	130
K^+	24
Ca^{++}	6
Mg^{++}	5
Total phosphate	5
Total salinity	*c.* 170 (as NaCl)

*p*H = 6·7

With this modification our worms are kept under the conditions previously described (Baldwin, 1943).

Preparation of muscle strips

When our work began there was nothing to indicate what kind of saline bathing medium might be suitable for maintaining the physiological condition of fragments

of isolated nematode tissue, and it was accordingly difficult to be sure whether, when a given preparation failed to show any lasting activity, the saline or the preparation itself was at fault. Occasionally, however, individual fragments of the body wall showed a more or less rhythmic activity that enabled us in time to develop the following operative technique. Large, active female worms were used throughout.

A suitable specimen is dropped from a height of about 12 in. on to the bench. This treatment leads to a sharp contraction of the musculature, immobilizes the animal for the time being, and considerably increases the firmness of the whole body, thus facilitating the procedure. It is essential as a preliminary measure since, if it is omitted, the final muscle strip usually contracts to a length shorter than the minimum which, in our experience, is necessary for satisfactory performance.

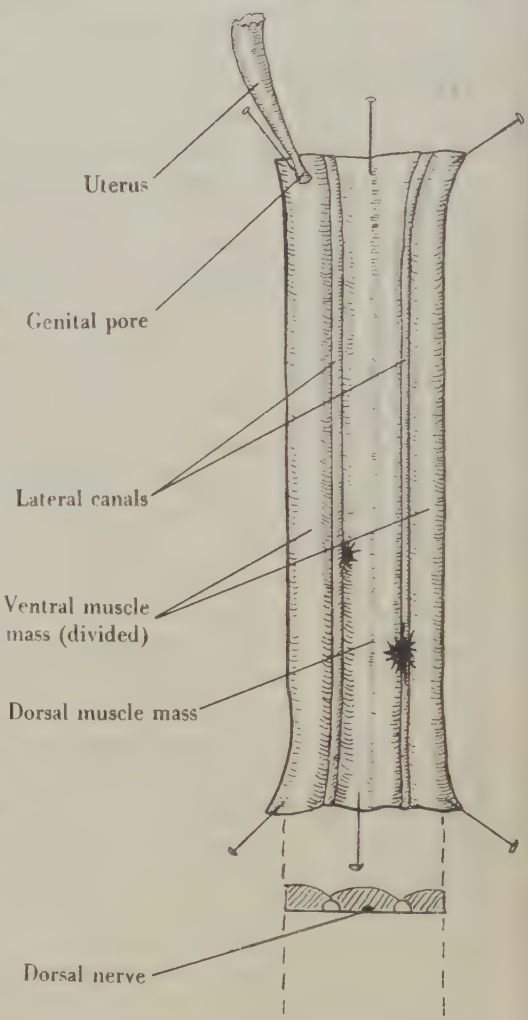

Fig. 1. See text for explanation.

The 'stunned' worm is pinned down to a cork mat, ventral surface upwards, by passing a pin vertically through the body about 5 mm. behind the genital pore. A second pin is passed through about 4 cm. in front of the pore and the external parts are cut away. The upper (ventral) muscle is divided by a longitudinal incision with a very sharp scalpel, the divided portions are deflected and pinned down, and the gut carefully removed with fine forceps. The preparation now has the appearance illustrated in Fig. 1. Further cuts are made along the lateral canals, great care being taken to avoid injury to the dorsal muscle. Silk ligatures are loosely applied to the strip of dorsal muscle thus isolated, the first being applied about 2–3 mm. in front of the level of the genital pore and the second 2·5 cm. farther forward. Variations of $\pm$ 1–2 mm. are immaterial, but the optimal length appears to be 2·5 cm. After tying the ligatures the remainder of the tissue is removed. Better results were obtained with loose than with tight ligatures, and it has been found necessary to leave rather long 'tails' (4–5 mm.) beyond the ligatures in order to avoid slipping.

Each muscle strip thus prepared consists essentially of a ribbon-like piece of dorsal muscle together with the corresponding dorsal and sublateral nerves. While the external surface of the strip retains its cuticle, the inner side is exposed. Strips of ventral muscle can be similarly prepared by starting with a dorsal incision. The region used corresponds to the 'intermediate preparations' of our earlier work; we have not so far attempted to obtain exposed fragments comparable with our 'anterior preparations'.

It is best to prepare several strips at a time. Each in turn is transferred to a bath at 38° C. containing the special buffered experimental medium described below (p. 286), a load of 20–40 mg. is applied to each and the whole batch is kept under observation. The immediate response to being placed in the warm bath consists in a powerful, long-lasting contraction, followed by gradual relaxation. After a period of relative quiescence, a more or less rhythmic activity sets in in about 60% of all strips prepared from a good batch of material, and full activity is usually established in 30–60 min.

It may be pointed out that different batches of worms yield preparations of very variable degrees of usefulness. This variability, we believe, is due to circumstances beyond our control, i.e. to the treatment accorded to the animals during collection and transport. It is particularly noticeable that very few successful preparations can be obtained if the temperature of the medium in which the worms are transported has fallen below 30° C. before reaching the laboratory.

Loading and recording

The movements executed by these preparations vary widely. They consist essentially of alternating longitudinal contractions and relaxations, but on account of the closeness of attachment of the muscle to the cuticular layer and the considerable rigidity of the latter, the strips often assume bizarre shapes in the contracted condition. The best preparations are selected for attachment to the recording apparatus.

A very light isotonic lever with a Gimbal-mounted writing point was used in all our later experiments, but in some of the early work we used simple levers with adjustable loads. It was found in many cases that, if the load is gradually increased, a critical point is reached at which the strip suddenly relaxes very sharply indeed and at once contracts again, leading in some specimens to the onset of vigorous activity. In some experiments we have seen these small strips of muscle working actively under loads of as much as 50 g. Later, however, we abandoned heavy loads entirely in favour of weights of only 20–40 mg.

A number of representative tracings are shown in Fig. 2. Individual strips give records that vary markedly in amplitude and frequency, but the behaviour of a given specimen is usually very consistent. Strips showing aberrant behaviour or any serious irregularity are usually rejected if they fail to settle down after 60–90 min. Particularly noteworthy is the typical difference between dorsal strips and those prepared from ventral muscle. This difference is remarkably consistent and must presumably correspond to some difference in physiological constitution, the precise

nature of which remains to be investigated. In the meantime the bulk of our experiments have been carried out on dorsal preparations, since a larger proportion of these gives useful behaviour on the kymograph.

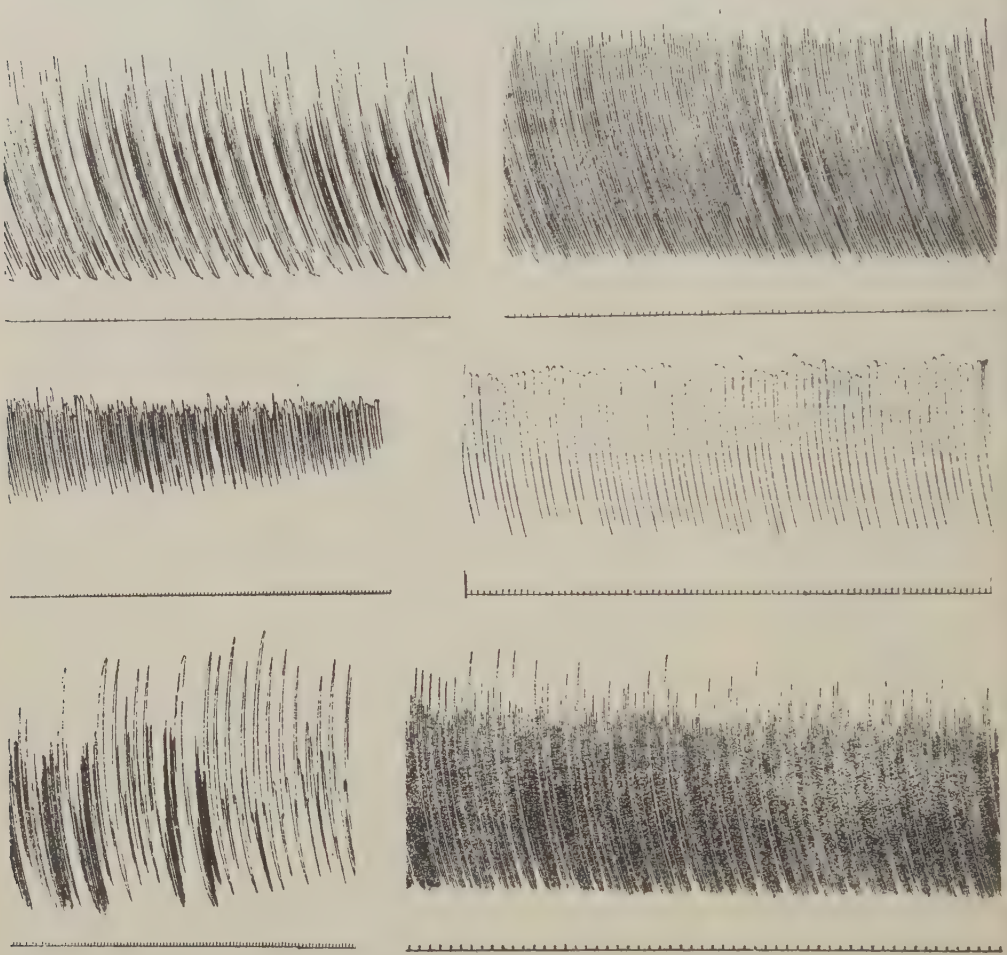

Fig. 2*a*. Typical tracings of behaviour of dorsal muscle strips. Upward stroke of lever corresponds to contraction; time-marker intervals, minutes in all cases. Read from left to right. Records taken in experimental medium (p. 286) unless otherwise stated.

Environmental conditions

(i) *General.* In the absence of much specific information about the general physico-chemical conditions prevailing in the normal internal and external environments of *Ascaris in situ* in the gut of the host, we had of necessity to make numerous empirical trials to find conditions under which the physiological activity of our preparations might be maintained. The conditions to be taken into account, apart from the relative ionic composition of the medium and the possibility of specific peculiarities, included temperature, *p*H, osmotic pressure, oxygen tension,

carbon dioxide tension and so on. A temperature of 38° C. was used throughout, this being the one factor of which the suitability seemed to be logically assured.

Bunge (1883, 1890) and Weinland (1901) are among those who have claimed that *Ascaris* can live under strictly anaerobic conditions. Slater (1925) severely criticized their conclusions on the grounds that the precautions taken to ensure complete anaerobiosis in their experiments were quite inadequate. That the tissues of *Ascaris* can utilize oxygen is certain, and there are in the literature many records of measurements of the Q_{O_2} of intact worms and of their several tissues under a variety of experimental conditions (e.g. Harnisch, 1933, 1935; Krüger, 1936). According to

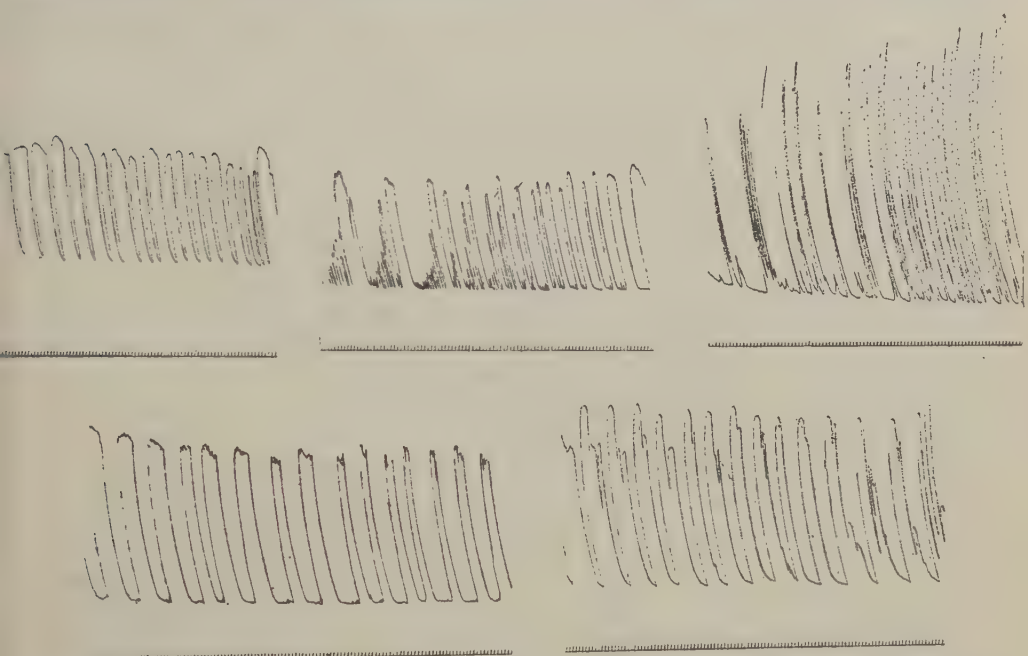

Fig. 2*b*. Typical tracings of behaviour of ventral muscle strips. Upward stroke of lever corresponds to contraction; time-marker intervals, minutes in all cases. Read from left to right. Records taken in experimental medium (p. 286) unless otherwise stated.

Laser (1944), the rate of oxygen consumption of the intact worm is not much smaller than that of intact mammalian organisms kept under comparable conditions of temperature and oxygen tension, though high oxygen tensions were found to have definitely toxic effects.

There is little reason to think that much oxygen is normally present in the habitual environment of *Ascaris*. Long & Fenger (1917), whose results have been substantially confirmed by von Brand & Weise (1932), reported that the intestinal gases of pigs contain variable amounts of oxygen, averaging about 5 %. The experiments of Bunge, Weinland, Slater and many others show, however, that this parasite can live for long periods at very low oxygen tensions and, in our earlier work, we have ourselves seen tubular fragments of *Ascaris* actively recording on the kymograph

after 24 hr. or more under experimental conditions in which no provision whatever was made for oxygenation of the medium.

We decided for our present experiments, therefore, to use cylinder nitrogen to provide the atmosphere in our media. Ordinary commercial nitrogen usually contains a few per cent of oxygen, and this, it was hoped, would suffice to meet the oxygen requirements of the tissues without exposing them to the danger of oxygen poisoning (see Laser, 1944). Our media were 'gassed' at the beginning of each experiment: maintenance of gassing throughout the experiments resulted in no improvement in the behaviour of our preparations, nor did frequent replacement of the medium by freshly gassed samples. Indeed, it has been our experience that, once a muscle strip has settled down to a steady pattern of behaviour, the less it is disturbed the better.

(ii) *Body fluid as experimental medium.* We anticipated that the properties and composition of the body fluid of *Ascaris* (Table 1) would provide a useful guide to the conditions required for maintaining the activity of our muscle strips. Several features of this body fluid call for special comment however.

The large disparity between total chlorides (53 m*M*) and total base (166 m*M*) is largely due to the presence of volatile fatty acids. Bunge (1890) long ago noticed the peculiar smell of media in which *Ascaris* have been kept, and attributed it to the presence of a volatile fatty acid. Weinland (1901, 1904) believed that a valeric and a caproic acid are concerned, and that these are essentially excretory products. Although these acids have been studied by a number of workers (e.g. Schulte, 1917; Flury, 1912; Krüger, 1936; von Brand, 1934), they have still not been satisfactorily identified. Much discussion has centred round the possibility that they might be formed, not by the worms themselves, but by bacteria, a possibility that could not easily be eliminated because of the virtual impossibility of sterilizing *Ascaris* itself. Most of the work hitherto carried out on these acids has been done on material collected, usually by steam distillation, from media in which *Ascaris* has been housed and in which, therefore, bacterial activity has probably or even certainly been considerable. Schimmelpfennig (1902), however, claimed that the same fatty acids are present in ethereal extracts of the whole worm, an observation later confirmed by Flury (1912).

We ourselves carried out a number of estimations on freshly collected body fluid previously deproteinized with tungstic acid. Aliquot portions of the filtrates were steam-distilled in a Markham (1942) apparatus, and the acids estimated in the distillates by titration with CO_2-free NaOH in a CO_2-free atmosphere. Added valeric acid was recoverable to the extent of 96–98 % under the conditions employed. The results obtained are presented in Table 2 and indicate the presence in the body fluid of some 50 m*M* steam-volatile fatty acids.

The fact that these acids are present in such large quantity in the perienteric fluid itself would seem to militate against the supposition that they owe their origin to bacterial activity and argue for them a biological role of considerable importance, though what this role may be is at present uncertain. It has usually been supposed that they are excretory products, but they may conceivably discharge an osmotic

role comparable with that of the urea which is so characteristic a feature of the blood and tissues of the Elasmobranchii (Smith, 1936) or with that of the glycine, taurine and other extractives which appear here and there in the animal kingdom, often in remarkably large concentrations (cf. Krogh, 1939, p. 196).

Together with chloride, these acids are equivalent to about two-thirds of the total base found by analysis. To what extent they are ionized is uncertain, the *p*H of the body fluid being somewhat unsure. The remaining anions have not been identified, but in view of the close resemblance between the compositions of the body fluid and the gut contents of the host, and of the probably high bicarbonate content of the latter, it seems likely that they must consist largely of bicarbonate.

Table 2. *Steam-volatile fatty acid content of body fluid of* Ascaris

Exp. no.	Days in laboratory	m*M* volatile acids
1	1	53
2	2	64
3	1	52
4	1	58
5	Fresh	50
6	,,	51
7	,,	35
8	,,	34
9	,,	57
10	,,	60
		Average 51

Table 3. *Relative composition of some biological and experimental fluids*

	Na	K	Ca	Mg	m*M* total base
Ascaris, body fluid	100	19	5	4	166
Pig-gut contents	100	21	11	4·8	171
Ringer's solution	100	1·7	1	0	116
Tyrode's solution	100	1·8	1·2	0·7	155
New 'keeping medium'	100	19	5	4	166
Experimental medium (p. 286)	100	3	1·5	3	120

Anions apart, the relative composition of the perienteric fluid resembles that of the external environment (i.e. the pig-gut contents) rather closely (Table 3) and differs markedly from that of the internal media of animals in general. Table 3 includes the relative ionic composition of Ringer's and Tyrode's solutions, which may be taken as roughly representative of the normal internal environment of animals as a whole. Because of the numerous known peculiarities of the Nematoda we were tempted to believe that the perienteric fluid of *Ascaris* might, in spite of its somewhat peculiar composition, represent the true *milieu intérieur* of that animal, and carried out many experiments in solutions made up to resemble this fluid in inorganic composition. We did not, however, attempt to imitate the fatty acid composition, the acids being still unidentified, but replaced them by chloride in preference to running the risk of introducing foreign anions of a possibly toxic nature.

Numerous strips became active in the 'synthetic body fluid', but in no case was the activity long maintained, and we finally came to the conclusion that the body fluid does not in reality correspond to the true *milieu intérieur*, using that term in its classical sense. Its true biological status awaits elucidation: conceivably it is, in essence, an excretory product.

(iii) *Experimental medium.* Media of the Ringer and Tyrode types seemed to offer the best prospects of success, and some hundreds of experiments in all were carried out in a search for the most satisfactory conditions. Magnesium was found to be a necessary constituent as, indeed, is usually the case for invertebrate tissues. For buffering purposes we decided on a mixture of carbon dioxide and bicarbonate, together with phosphate, since the combination gave more satisfactory results than either alone.

We are indebted to Prof. Hobson for informing us that 'the *p*H of the body fluid is rather uncertain owing to the fact that it changes very rapidly on exposure to air. When taken with a glass electrode as quickly as possible after "bleeding" it seems to average about 6·8, but I have known it as low as 6·5 and as high as 7·0' (personal communication). Probably the drift observed in these determinations must have been due to the loss of volatile fatty acids when the fluid was exposed, and we believe that the *p*H of the body fluid *in situ* must probably lie nearer to 6·5 than to 7·0. Our muscle strips, however, seem to be little affected by changes of *p*H between 6·5 and 7·5, and we settled finally on a *p*H of 7·1 as a matter of experimental convenience.

The osmotic pressure of the body fluid likewise proved no guide to the total salinity desirable in an experimental saline. Muscle strips prepared in the usual manner were suspended in a series of media having the same relative ionic composition but differing in total salinity, and were weighed at intervals. At 120 m*M* the changes of weight were usually within 5% of the initial value over periods of 3 hr., larger changes being observed at higher and at lower osmotic pressure. We therefore determined to use a total salinity of 120 m*M*.

It is not necessary here to give a detailed account of the experiments carried out with media of various compositions. Our aim was to find, if possible, an experimental medium in which rhythmic activity could be developed and subsequently maintained on a steady base-line, and eventually we adopted a medium, the preparation of which is described below, having the following properties:

$Na^+ : K^+ : Ca^{++} : Mg^{++} = 100 : 3 : 1{\cdot}5 : 3$,
$HCO_3^- = 6$ m*M*,
Total phosphate = 3 m*M*,
Total salinity = 120 m*M* (as NaCl),
*p*H = 7·1,
Thiamine hydrochloride = 1 : 10,000.

In this medium we were able to maintain the activity of our strips for periods up to 9 hr., with a usual survival of 5–8 hr. Longer survivals were not obtained under any of the numerous conditions tested, but, having due regard to the nature of the

muscle strip which, unlike the isolated frog heart for example, is in no sense an intact physiological unit, we feel that these results may be regarded as satisfactory.

The incorporation of thiamine into the medium followed an observation that this vitamin encourages relaxation of the muscle strips. Many preparations show a marked tendency to remain contracted for relatively long periods, relaxing only infrequently and partially. The general impression gained by careful observation of preparations of this kind was that some sort of nervous dysfunction was involved and, recalling the polyneuritis associated with vitamin B_1 deficiency in other animals, we tried the effect of adding small amounts of thiamine to the medium. A typical record is shown in Fig. 3. The type of behaviour in the early part of the record is far from rare in these preparations. The dramatic effect of adding thiamine is well shown in the figure: almost immediately the strip begins to relax more fully and more frequently and after a brief interval a smooth, long-lasting activity is attained.

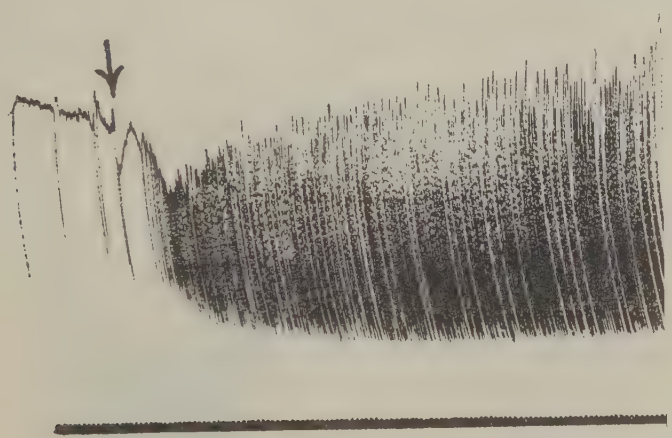

Fig. 3. Effect of thiamine on muscle strip. Thiamine added at arrow.

After careful consideration we have come to the conclusion that the routine addition of a small amount of thiamine to our experimental saline is justified, and that the activity elicited in this way must be regarded as physiological. Other experiments have been carried out with the addition, together and separately, of members of the vitamin B_2 complex, including riboflavine, nicotinic amide, pantothenic acid, pyridoxin and *p*-aminobenzoic acid, but in none of these was there any change in the behaviour of the muscle.

We also tried the effect of adding small amounts of thiamine to our 'keeping medium' in the hope that the worms might be maintained in better functional condition. Their general appearance and activity seemed to be appreciably improved, but a series of survival experiments showed that the average life period is not increased by thiamine. The cause of death of *Ascaris* in laboratory media is not known: starvation alone seems unlikely to be the cause, for the tissues are still rich in glycogen at death. By analogy with what is known about the culture of parasitic micro-organisms it seems reasonable to assume that the isolated nematode dies in

culture because it depends upon its host, in the ordinary way, for day-to-day supplies of some essential nutrient or nutrients, but our results with *Ascaris* seem clearly to indicate that, whatever this material may be, it does not consist of thiamine alone.

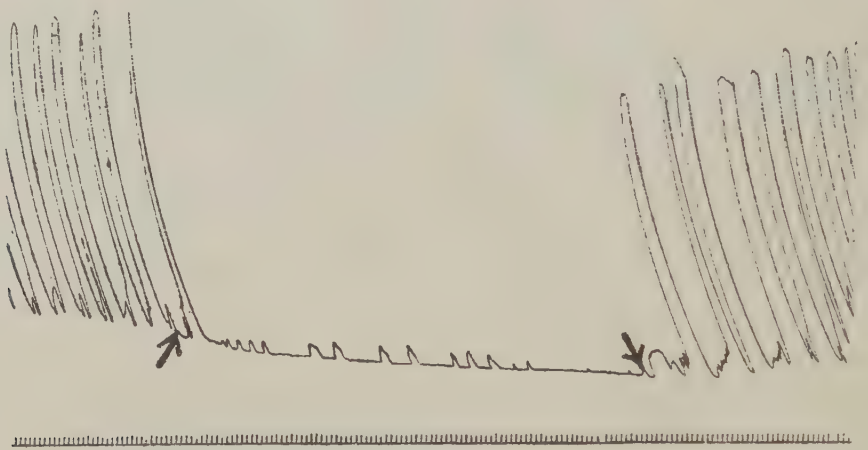

Fig. 4*a*

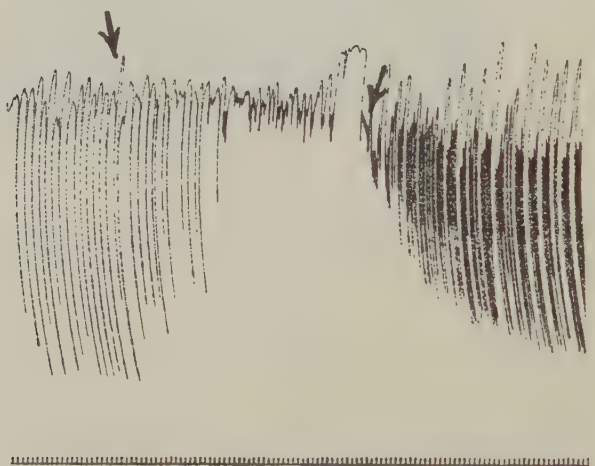

Fig. 4*b*

Fig. 4. Influence of changes in osmotic pressure of medium. Both records start at 120 m*M*, (*a*) decreased to 80 m*M* at first arrow, returned to 120 m*M* at second arrow; (*b*) increased to 150 m*M* at first arrow, returned to 120 m*M* at second arrow.

Preparation of the experimental medium

(i) *Stock solutions.* Molar solutions of NaCl, KCl, $CaCl_2.6H_2O$ and $MgCl_2.6H_2O$ are kept as stocks, together with *M* $NaHCO_3$ and 120 m*M* $NaH_2PO_4.H_2O$ and $Na_2HPO_4.2H_2O$. All these are prepared from A.R. reagents and made up in glass-distilled water. All subsequent dilutions are carried out with glass-distilled water.

(ii) *Working solutions*. The working solution of Na^+ is prepared by taking 117 ml. *M* NaCl, adding 7·5 ml. 120 m*M* NaH_2PO_4 and 17·5 ml. 120 m*M* Na_2HPO_4 and making up to 1 l. 50 ml. of the product are replaced by 50 ml. freshly diluted 120 m*M* $NaHCO_3$. This forms a buffered working solution containing 120 m*M* Na^+.

The remaining solutions are freshly prepared by dilution of the molar stocks to 120 m*M*. 30 ml. KCl, 15 ml. $CaCl_2$ and 30 ml. $MgCl_2$ (120 m*M* in each case) are added to each litre of the solution of Na^+, followed by the addition of 100 mg.

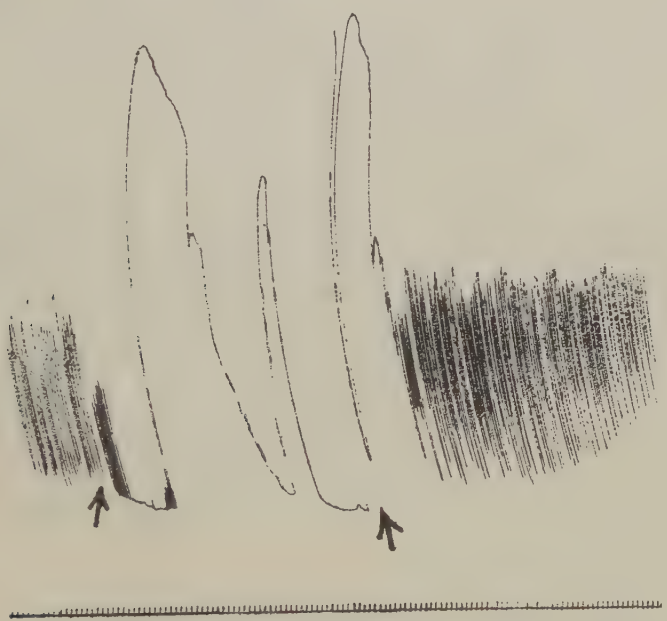

Fig. 5. Influence of changing from normal experimental medium to 'synthetic body fluid' (first arrow) and return to normal (second arrow).

thiamine hydrochloride. Finally, the mixture is gassed with 5 % carbon dioxide in 95 % nitrogen from a cylinder, no steps being taken to remove traces of oxygen. The gas mixture is bubbled through in the form of a very fine spray until the solution reaches a *p*H of 7·1 as judged by testing a sample against bromthymol blue (*p*H = 7·1). The gas stream may then be reduced but should not be cut off completely, since the dissolved gases tend to escape slowly from the solution.

Variations on this standard composition can readily be made by adding different amounts of KCl, $CaCl_2$, etc., while the *p*H can be modified by adding less or more of the $NaHCO_3$ or by varying the proportions of the two phosphates.

DISCUSSION

Many experiments have now been performed on muscle strips prepared in the manner and under the conditions described here. Experiments on the pharmacology of the muscle strips will be presented in a later paper: for the present it may be stated that these preparations are capable of giving useful indications of the influence of various drugs with anthelminthic and other properties and that the results obtained are, in the main, freely repeatable.

As illustrations of the reactions of the strips to unfavourable conditions in their immediate external environment, reference may be made to Fig. 4, which shows the effect of changes in osmotic pressure, and to Fig. 5, which shows the effect of changing from the standard experimental medium to 'synthetic body fluid' and back again.

SUMMARY

1. A technique is described for the preparation from the body wall of *Ascaris* of semi-isolated strips of muscle. These strips are exposed on one side to the surrounding medium and are suitable for studies of the action of anthelminthic and other drugs upon the exposed musculature.

2. A medium suitable for use in such experiments has been devised and its preparation is described.

3. Media made up to represent the body fluid of *Ascaris* fail to support physiological activity in the exposed muscle strips, and it seems that this perienteric fluid does not correspond to the true *milieu intérieur* of this nematode.

4. Some new observations on the nature and composition of the perienteric fluid are presented incidentally in the text.

The authors are indebted to the Agricultural Research Council for a grant during the tenure of which this work was carried out. The expenses were defrayed by a further grant from the Council. We are indebted to other members of the Council's nematode team for much advice and information, to Dr W. Feldberg for criticisms and suggestions, to Mr W. B. Edwards for preparing the drawing reproduced in Fig. 1, and to Prof. A. C. Chibnall, F.R.S., for his interest in the work. The assistance of Miss Marjorie Cotton in the early stages of the work is also gratefully acknowledged. Particular thanks are due to the Manager of the St Edmundsbury Co-operative Bacon Factory, who made the work possible by his unfailing courtesy in making arrangements for the collection and speedy transport of material to the laboratory.

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THE RESPONSES OF ONION AND LILY MITOSIS TO COUMARIN AND PARASORBIC ACID

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(With Plate 10)

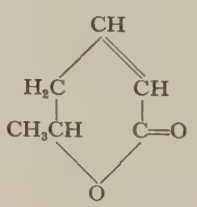
A substance which shows comparable ontogenetic effects throughout the plant and animal kingdoms is valuable in interpreting certain fundamental growth phenomena. Colchicine is such a compound. Parasorbic acid is another, and is of even greater interest since it exhibits a range of effects far wider than that of colchicine.

Parasorbic acid inhibits the growth of Amphibia and mammals (Heaton, 1929), destroys the mesenchyme of planarians (Hauschka, Toennies & Swain, 1945), and in tissue cultures inhibits the mesenchyme of embryonic chick (Medawar, Robinson & Robinson, 1943; Kuhn *et al.* 1943) and of mammals (Drew, 1927). It also inhibits the fermentation of yeast, the germination of pollen, and the germination of seeds (Kuhn *et al.* 1943; Köckemann, 1936). Parasorbic acid fed to rats has been shown to inhibit the Jensen sarcoma (Heaton, 1929), but *in vitro* evidence as yet is inadequate to demonstrate whether it selectively inhibits malignant fibroblasts (Cornman, 1945). Kuhn and his co-workers have shown that it does not attack Erlich carcinoma in tissue culture.

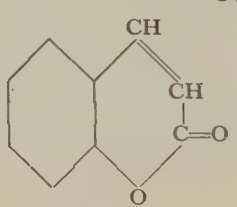
The action of lactones such as parasorbic acid, in common with that of some antibiotics, may possibly be explained by their ability to react with sulphydryl groups (Cavalito & Haskell, 1945).

In the above experiments the parasorbic acid was obtained from yeast, fruits, and animal tissues. Growth inhibitors are often reported in the literature (*Amer. J. Bot.* **31**; suppl.), which may also prove to be related to parasorbic acid, extending even further its natural range of occurrence.

The related compound, coumarin (see diagram), occurs in a number of plants, including sweet clover. It also has growth and germination-inhibiting powers (Kuhn



parasorbic acid



coumarin

et al. 1943; Nutile, 1945), which Veldstra & Havinga (1943) relate to its antagonism toward naphthaleneacetic acid. It may not be purely an inhibitor, however, inasmuch as Grace (1938) finds it can function as an auxin.

The purpose of the work presented here was to test these two compounds and sorbic acid, the straight-chain isomere of parasorbic, for their influence on mitosis. In addition to whatever of interest is revealed in the mitotic effects themselves, it should help to determine the role of mitotic inhibition in the mechanism by which normal and malignant growth is slowed by these compounds.

METHOD

Roots growing from bulbs of *Allium cepa* and *Lilium longiflorum* provided the dividing cells. The roots were sprouted in tap water, then immersed in saturated solutions of coumarin, sorbic acid, or crude parasorbic acid.

Two to eight roots were fixed after $2\frac{1}{2}$, 5, $11\frac{1}{2}$ hr. exposure and after 9, 12, 31 and 45 hr. in tap water following $11\frac{1}{2}$ hr. exposure. Zenker's and Bouin's fixatives were used, followed by routine paraffin sectioning at 8 and 10 μ and staining in Harris's or Heidenhain's iron haematoxylin.

OBSERVATIONS

Growth effects

Exact measurements of growth were not made, but it was noted that elongation of onion roots continued only a short time after immersion in the experimental solutions. The vapour from a saturated solution of parasorbic did not prevent the unsubmerged roots from growing into the solution.

Hypertrophic swellings were visible on the coumarin-treated roots after $32\frac{1}{2}$ hr., that is, after 21 hr. recovery. Smaller swellings were induced by parasorbic acid after 31 hr.

Cytological effects of coumarin

After $2\frac{1}{2}$ hr. the mitotic block was complete. Some telophases with phragmoplast and cell plate and some anaphases remained (see Table 1). All metaphases appeared to be blocked. Abnormalities included telophase cells with two nuclei or double (dumbbell) nuclei, and the typical dispersion and shortening of metaphase chromosomes. These all resulted from the destruction of the metaphase and anaphase spindles.

After 5 hr. the picture was that of a complete colchicine effect. Blocked metaphases predominated, and prophases were the only other mitotic stages present. The metaphase chromosomes had shortened further (Pl. 10, fig. 2), and in some cases had split to form the *X*-configuration typical with delayed centromere division, or the 'ski-pairs' of complete splitting (Pl. 10, fig. 3).

During these first 5 hr. of treatment, reversion to interphase progressed more slowly than the evolution of prophases, resulting in an accumulation of metaphases. The blocked metaphase chromosomes became packed into dense masses (Pl. 10, fig. 4, lower right) before forming giant interphase nuclei. This aggregation was the only morphological variance from the colchicine response wherein the chromosomes remain scattered. Nuclei with projections or lobes were frequent, but unequal nuclei or karyomeres were rare, and only found after a period of recovery (Pl. 10, fig. 5), during which a second abortive division may have produced a fragmented

nucleus. Reversion of blocked anaphases produced double nuclei connected by bridges of varying thickness (Pl. 10, fig. 4) and binucleated cells. These cells with doubled chromatin content were enlarged to varying degrees.

Coumarin slowly suppressed the initiation of mitoses, with the result that after 11½ hr. only an occasional prophase or a blocked metaphase with extremely short, thick chromosomes could be found.

Table 1

Under the heading 'binucleate' are included cells where the anaphase groups separated, but subsequently formed nuclei joined by chromatin bridges.

	Coumarin (exposure in hr.)				Parasorbic acid (exposure in hr.)		
	0	2½	5	11½	0	2½	5
Late prophase	44	90	70	23	93	40	59
Metaphase	43	137	89	56	76	51	43
Anaphase	19	7	0	0	29	33	16
Telophase	28	3	0	0	24	15	8
Binucleate	0	45	26	26	0	0	0

The figures in Table 1 record all mitoses in the middle sections, totalling 100 μ, of a typical root from each group (in the last column a block 700 μ thick was counted to compensate for the scarcity of figures). These numbers cannot be regarded as a statistical approach, inasmuch as the counts vary with the size and mitotic activity of the individual root. Nevertheless, the trend in the proportions of mitotic phases is unmistakable. Once treatment has begun, the metaphases exceed the late prophases, while anaphases and telophases decrease and disappear after 5 hr. These later stages are numerically replaced by binucleate cells, a convincing demonstration that they did not complete the mitotic cycle, but reverted to interphase without completing anaphase separation (dumbbell-shaped fused nuclei) or partitioning of the cell (binucleate). It may be significant that the number of binucleate cells progressively decreased during treatment and recovery, suggesting that fusion and rounding up had produced one symmetrical nucleus.

Recovery in onion

Onion roots treated for 11½ hr. and subsequently returned to tap water did not renew mitotic activity within 9 hr. After 21 hr., many diploid mitoses were to be found. After 45 hr., polyploid (Pl. 10, fig. 6) and binucleate cells had also resumed division, many of them with poorly developed spindles and lagging or scattered chromosomes. These secondary irregularities may have been the cause of the unequal nuclei in such hypertrophied cells as that shown in Pl. 10, fig. 6. At this time some roots still showed no mitotic activity, and the pycnotic nuclei and highly acidophil cytoplasm of some cells suggested a dead or dying condition.

Lily

The course of events in lily roots was similar to that in onions. Blockage of mitosis was complete after 2 hr., and after 3 hr. an accumulation of metaphases was observed along with the other concomitants of interrupted and reverting mitoses: short

chromosomes divided into parallel pairs (Pl. 10, figs. 8, 9, lower), tetraploid chromosome groups probably derived from anaphases (Pl. 10, fig. 7), giant lobed (Pl. 10, fig. 10, upper right) or double nuclei (Pl. 10, fig. 10, lower left), bridged nuclei, and binucleate cells. All showed varying degrees of irregularity in the nuclear outline, reflecting a moderate scattering of chromosomes. Blocked metaphases reverted to interphase via densely massed chromosomes (Pl. 10, fig. 9, upper) as in the onion. After 6 hr. the suppression of mitosis was evidenced by a decrease to a few prophases and blocked metaphases. Recovery of the lily cells has not been studied.

Cytological effects of parasorbic acid in the onion

Parasorbic acid has little in common with coumarin in its effects on the onion-root mitosis except that counts show a slight excess of metaphases which may represent an accumulation of metaphases or a relatively more rapid decrease of prophases (see Table 1). This altered ratio of mitotic phases was found after $2\frac{1}{2}$ and 5 hr. The metaphase chromosomes were noticeably shortened (Pl. 10, fig. 1). It must be emphasized, however, that normal anaphases and telophases persisted and these phases occurred in all layers, so unequal penetration cannot explain their persistence. There were no transitional phenomena indicating a reversion of metaphases to interphases. In roots exposed for longer periods, no giant nuclei were found; and in roots allowed to recover, no tetraploid mitoses could be detected. It would seem that the progress of mitosis had been slowed but not stopped, and that the spindle mechanism continued to function. One hesitates to ascribe so slight an effect to the parasorbic acid, inasmuch as only a crude preparation was available, and in a saturated solution enough impurity may have been present to alter the speed of mitosis.

Few mitoses remained after 5 hr. exposure, and only a rare prophase or metaphase after $11\frac{1}{2}$ hr. An occasional prophase was to be found after 9 hr. recovery. Mitoses in appreciable numbers did not reappear until 31 hr. in tap water had elapsed. At this time all stages were visible in the more vacuolated cells at the base of the meristem, while prophases and a few metaphases and anaphases were found in the apex. After 14 hr. more, mitoses of all stages were proceeding normally throughout the proliferative tissue.

Effects of sorbic acid in the onion

Saturated sorbic acid immediately stopped growth and caused the roots to become flaccid. Mitoses in these roots were normal in all parts of the meristematic region after 6 hr. exposure, and later after 5 hr. recovery. The excessive vacuolization of the cytoplasm was similar in appearance to that caused by poor fixation. Indeed, since acidity of even half-saturated sorbic acid is *p*H 4·47 (glass electrode), its only effect upon the cell was probably an immediate coagulation. Cytological data for the half- and quarter-saturated acid solutions are not yet available, but at quarter-saturation the roots lost their turgidity within 5 hr.

DISCUSSION

Coumarin takes its place among the natural mitotic poisons, foremost among which are the alkaloids and the essential oils. Others of these aromatic substances are anethol (Lefèvre, 1940), apiol (Gavaudan & Gavaudan, 1940), thymol (Villars, 1940), and methyl methylanthranilate (Simonet & Igolen, 1940). In connexion with the problem of a plant's protecting its growth processes from its own poison, it would be interesting to ascertain whether clover, anise, parsley, thyme and the orange, like *Colchicum* (Cornman, 1942), are sensitive to high concentrations of these active agents contained in their own cells, and whether there is any cross-immunity. Such an investigation might suggest an explanation of the fact that benzene derivatives are able to produce identical effects in a cell (benzene and colchicine represent the extremes), while a slight change in a molecule (colchicine to colchiceine by loss of a methyl group) produces a thousand-fold change in potency (Ludford, 1936).

Coumarin and parasorbic acid differ by one benzene nucleus, and as has been repeatedly shown, the benzene molecule is particularly efficacious in blocking mitosis. The germination-inhibiting properties, on the other hand, appear to depend upon the lactone ring, since both compounds prevent seed germination.

Animal cells respond to parasorbic acid and in a particularly interesting manner. In tissue culture, for instance, the fibroblasts are inhibited while epithelial cells continue to proliferate. Now in well-nourished tissue cultures the fibroblasts outgrow epithelial cells, so one might suspect that parasorbic acid acted by disrupting mitosis, thereby damaging the more frequently dividing cells. The above observations show that such is not the case, if we may generalize from plant cells (onion and lily root meristems have repeatedly been shown to be efficient detectors of poisons which also block animal mitosis). It remains possible, however, that there may be something significant in its preventing the onset of karyokinesis, although this is a property of most mildly toxic agents.

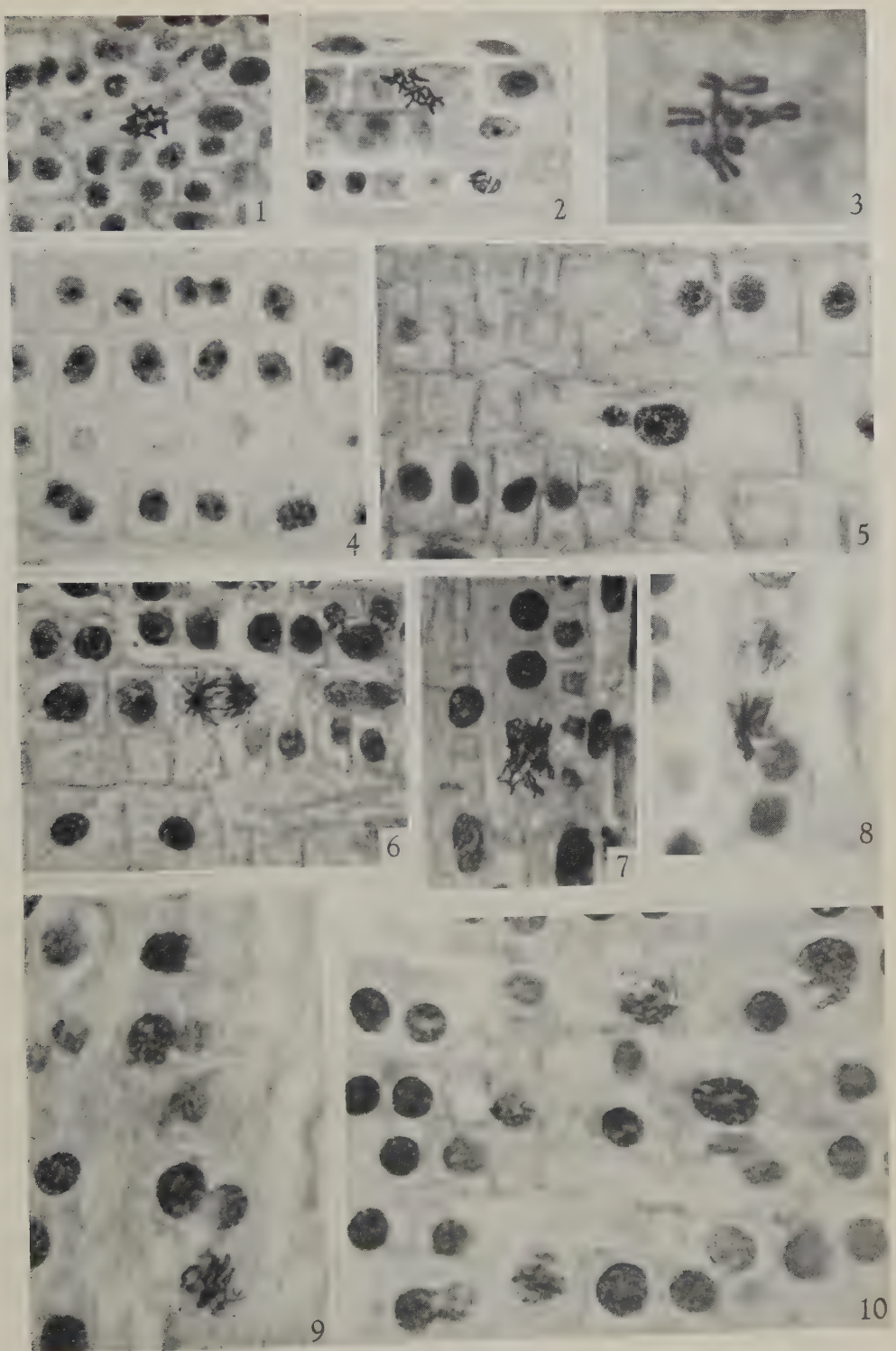
Mitotic poisons have also been suggested for the chemotherapeutic treatment of cancer. It may prove valuable to determine whether the therapeutic value of parasorbic acid, as demonstrated with the Jensen rat sarcoma, could be enhanced by endowing the molecule with anti-mitotic activity, guided by the pattern of the coumarin molecule.

SUMMARY

1. In *Allium* and *Lilium* roots, saturated aqueous solution of coumarin produced a disruption of the metaphase typical of many benzene derivatives, viz. suppression of the spindle, splitting and shortening of the chromosomes, with retarded division of the centromere. The resultant polyploid nuclei and binucleate cells resumed division when the roots were returned to water.

2. Saturated solution of parasorbic acid slowed *Allium* mitosis, but caused no abnormalities.

3. Both coumarin and parasorbic acid eventually prevented the inception of mitosis, and this suppression of prophases persisted for several hours after removal of the agent.



CORNMAN—RESPONSES OF ONION AND LILY MITOSIS TO COUMARIN AND PARASORBIC ACID

4. The results are in agreement with prior evidence that in some configurations the benzene ring disrupts mitosis whereas the lactone ring inhibits growth.

I am indebted to Dr John J. Biesele and to Dr C. W. Metz for suggestions regarding this manuscript. The lily bulbs were kindly supplied by Dr S. L. Emsweller of the U.S. Department of Agriculture. All three compounds were generously contributed by Dr Werner Bergmann.

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EXPLANATION OF PLATE 10

Fig. 1. Onion parasorbic acid, 5 hr. exposure. Short disoriented chromosomes of the slowed metaphase.

Figs. 2–4. Onion, coumarin, 5 hr. exposure. Fig. 2. Shortened, moderately scattered metaphase chromosomes with incipient split. No spindle remnant. Fig. 3. Split chromosomes with short, thick chromatids still attached at the centromere. Fig. 4. Restitution nuclei, one from a metaphase (lower right) showing densely packed chromosomes just reverting to interphase, and two from anaphases, showing bridges of different widths.

Figs. 5, 6. Onion, coumarin, 11½ hr. exposure—45 hr. recovery. Fig. 5. Hypertrophied cell with giant nucleus and karyomere. Fig. 6. Polyploid anaphase.

Figs. 7–10. Lily, coumarin, 3 hr. exposure. Fig. 7. 4*n* tangle of chromosomes probably arising from an interrupted anaphase. Fig. 8. Thick, split, clumped chromosomes of the blocked metaphase. Fig. 9. Blocked metaphase (below): chromosomes in the optical plane show the split but little contraction. Above is a blocked metaphase with the chromosomes tightly packed, just prior to reversion to interphase. Fig. 10. Double nucleus from a blocked anaphase (lower left). Lobed nucleus from a blocked metaphase in which the chromosomes were scattered.

The course of transformations in the blocked metaphases of both lily and onion is exemplified by the sequence: 9 lower, 2, 3 and 8, 9 upper, 4 lower right, 10 upper right, 5 (or more typically a uninucleate giant cell consonant with less scattering of the chromosomes), 6. Magnification photography by Lew Sunny.

THE OXYGEN CONSUMPTION OF *GASTEROSTEUS ACULEATUS* L. IN TOXIC SOLUTIONS

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(With Ten Text-figures)

INTRODUCTION

The respiratory exchange of entire organisms, isolated organs and tissues is a widely explored field in physiology, dating back to the classic work of Lavoisier, and in recent times still presents fresh problems for study, as, for instance, the work of Davis & Fraenkel (1940) on the oxygen consumption of flies during flight. Literature prior to 1916 is reviewed and catalogued in the monograph by Krogh (1916), since then the principal work dealing with the respiration of fishes appears to be that of van Dam (1938). This and other previous work on the respiratory exchange of fishes has been mainly occupied with the design of apparatus, the effects of variation in oxygen and carbon dioxide tension, and of temperature, age, size and starvation; and though in recent years river pollution problems have stimulated the study of the toxicity to fish of a vast range of toxic substances (see Ellis, 1937) there has been surprisingly little study of the effect of dissolved toxic substances on fish respiration. Powers (1917) made the first comprehensive study of the effect and degree of toxicity of a wide range of toxic substances to *Carassius* and (1922) investigated the effect of variations in the *p*H of the medium on the physiology of respiration in fishes. Carpenter (1927, 1930), in investigations of the effects of metallic pollution, concluded that the death of fish in dilute solutions of heavy metals is due to asphyxia consequent upon the metal ion precipitating the mucus upon the gill filaments, thus preventing their movement and impeding gas exchange; but, apart from one experiment (Carpenter, 1927, p. 385) in which it was shown that *Gasterosteus* immersed in a lead nitrate solution evolved carbon dioxide at rather less than 40% the normal rate, and the general observation that the opercular movements of fish so treated were more rapid than normal, Carpenter submitted no detailed evidence of the extent and rapidity of this asphyxiation effect. The present work was begun as a general study of the effect of toxic solutions on the respiration of fish and deals with heavy metal salts, chloroform and hydrogen cyanide and hydrogen sulphide.

APPARATUS AND METHOD

Compared with aquatic invertebrates fish consume oxygen rapidly and the general scheme for measuring their respiration rate usually includes a respiration vessel in which the fish is confined and through which water flows at a controlled velocity; its oxygen content on leaving the respiration chamber is compared with that of the

inflow. A very elaborate apparatus of this type is described by Wells (1935) in his paper on the relation between rate of respiration and temperature in *Fundulus*. In an earlier paper (1932) the same writer has shown that the metabolism of fish placed in such an apparatus is at first abnormally high, due to excitement on handling and the strange environment, and does not fall to what Wells calls 'normal' metabolism until at least 24 hr. after installation in the respiration vessel. His results bear this out, but it is obvious that the metabolic rate so determined must represent 'basal' rather than normal metabolism.

In toxic solutions fish always display a moderate or greatly increased degree of activity until they are largely overcome by the poison, and there is not much object in comparing their oxygen consumption in these solutions with that measured at complete rest. Elaborate apparatus for determining basal metabolism therefore

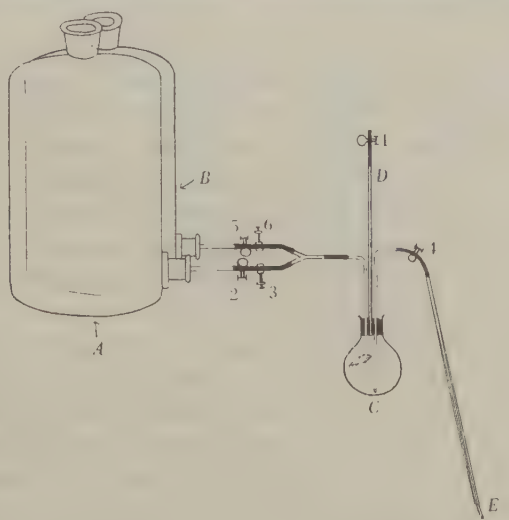

Fig. 1. General scheme of apparatus. *A*, *B*, 10 l. aspirators; 1, 2, 4 and 5, pinch clips; 3 and 6, screw clips. Other details in text.

serves no useful purpose, and a simple apparatus with which the oxygen consumption of the fish may be measured when the animal displays a normal degree of activity is more suitable. The general scheme of the apparatus used by the writer is shown in Fig. 1. An extraction flask of convenient size is used as respiration vessel; 150 ml. is a suitable size for a single large stickleback, a larger flask can be used for experiments in which a number of fish are placed together, and the smaller flask can be used for five or six small fish. The general experimental method will be understood from the following example in which a single fish was used, the solution in the case being 0·002 *N* $CuSO_4$.

The temperature of the room is maintained at 17° C. Some hours before beginning the experiment a suitable subject has been placed in a small aquarium on the experiment bench in order for it to become accustomed to the temperature, lighting conditions, etc. The flask *C* is detached, immersed in the aquarium, the fish gently

guided into it, and replaced. Water at room temperature saturated with air is now run into *C* from aspirator *A*, clip 4 being closed and clip 1 on the capillary tube *D* open to allow air to escape. When all air is expelled and *C* is full clip 1 is closed and clip 4 opened so that the water runs through *C* at 200–300 ml./min., this regulated by screw-clip 3.

The water is run through the respiration flask until the fish is accustomed to its new surroundings and remains still or swims around idly, its rate of opercular movement settling at 100–120/min. A sample of the water flowing out at *E* is taken and then clips 4 and 2 are closed, stopping the flow of water. The stop-clock is started as the clips are closed and the oxygen content of the sample determined by the Winkler method. With one fish in the respiration flask and the water flow 200 ml./min. the oxygen content of the outflow does not differ from the supply in the aspirator by more than 0·5%.

After 10 min. clip 1 is opened to allow air to enter, and by opening 4 a water sample is rapidly drawn off at *E*; clip 2 is kept closed. The taking of this sample is so arranged that the sample bottle becomes completely filled exactly at the end of the 10 min. period. The difference between the oxygen content of this sample and that of the first gives us a measure of the amount of oxygen used by the fish in the 10 min. interval during which the water flow was stopped. *C* can now be refilled from *A*, water run through for a few minutes and a second estimation made. A number of successive determinations with the same fish usually do not differ appreciably unless the degree of activity of the fish is greatly altered.

After a satisfactory measure of the normal oxygen consumption has been obtained most of the water is run off through *E*, *C* is filled with the copper sulphate solution from *B*, the stop-clock is restarted as the respiration flask is filled, the flow of the solution is stopped for the 5–15 min. time interval, and the difference in the oxygen content of samples taken at *E* at the beginning and end of this period compared with the previous difference noted with water give us a measure of the oxygen intake in the toxic solution in percentage of normal. It is not necessary to work out the actual oxygen consumption; the normal value for large sticklebacks in well aerated tap water at 17° C. is approximately 0·23 c.c. O_2/g./hr.

The solution is set flowing through *C* again, stopped for the 20–30 min. time interval, and the difference in oxygen content of samples taken before and at the end of this period gives a second point on the graph. Similarly, the oxygen intake in percentage of normal may be measured for 35–45 min., 50–60 min. and subsequent time intervals until the fish dies or the experiment is discontinued for some other reason.

These time intervals were not used invariably. In every graph the plotted points stand opposite the middle of the time interval over which the respiration rate was measured. Thus when the times chosen were 2–10, 12–20 and 22–30 min. (i.e. refilling and flushing *C* with fresh solution took 2 min.) the values for the oxygen consumption percentage are opposite 6, 16 and 26 min. on the time axis.

When using a 150 ml. respiration flask 120 ml. samples were drawn off and the iodine titration performed with *N*/30 thiosulphate in a 5 ml. micro-burette or

N/300 thiosulphate in a 50 ml. burette. Care was taken that the oxygen content of the solution in *B* was approximately the same as that of the water in *A*, both aspirators being filled, aerated and placed in position 12–24 hr. before use. Also the experimental conditions were always so adjusted that the oxygen content never fell below 70% saturation, at which level, according to van Dam (1938, p. 73), utilization in the trout is maintained at the normal value of 80%.

CHLOROFORM

The first respiratory depressant studied was chloroform. High concentrations are very toxic; a stickleback placed in a 1/1000 v/v solution ceases breathing and dies almost immediately. At somewhat lower concentrations the effect on the respiration rate can be followed, and three typical results are given in Fig. 2.

In a 1/3000 solution the respiration rate falls rapidly; almost immediately the fish begins to swim in a helpless, drunken fashion and in 8–10 min. sinks to the bottom of the flask where it lies still, with slow and flickering respiratory movements, until breathing ceases in about 20 min. In a 1/4000 or 1/5000 solution much the same sequence of events occurs, but in a slightly weaker solution still (14/100,000) something more like true anaesthesia is produced. At this concentration the fish struggles furiously for 20–30 min., gradually becomes quiet, and then sinks to the bottom of the flask, where it rests in a natural erect position, propped on its tail and pelvic spines. It now becomes almost rigid, its eyes set in a fixed stare, and the respiratory movements, previously irregular and flickering, are now of enormous amplitude, regular, but much slower than normal (28–32/min.), while the oxygen intake has fallen to about 30%. In this condition the fish may remain some time; one fish was kept in a 14/100,000 solution for 90 min. and revived rapidly on restoring the flow of water.

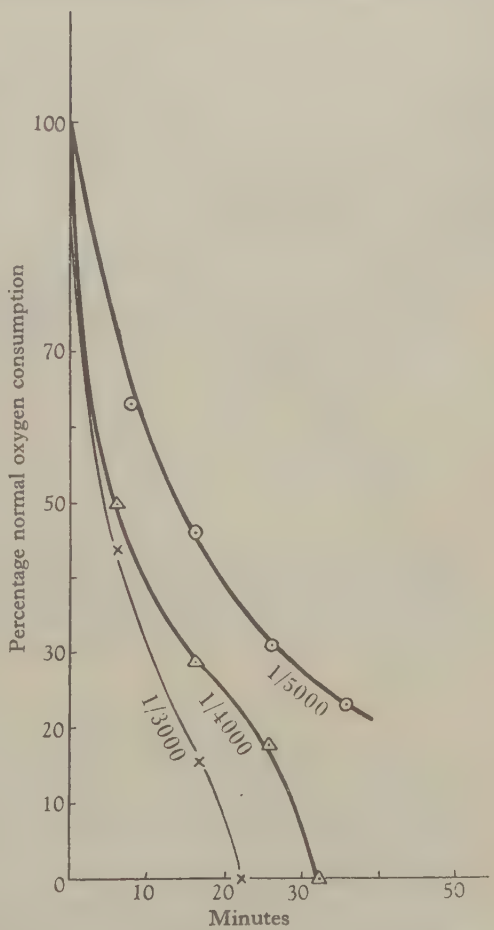

Fig. 2. Oxygen consumption curves for *Gasterosteus* in chloroform solutions. The concentrations are v./v.

Solutions appreciably weaker do not produce anaesthesia, but seem to excite the fish which struggles more or less continuously, the respiration rate rising to 120%

normal or more. The opercular movements become a little more rapid than normal, and of increased amplitude.

The theory that anaesthesia results from depression of the rate of tissue metabolism is a very old one, and this, and other theories of anaesthesia and narcosis are discussed by Heilbrunn (1943, p. 514 et seq.). It is evident that chloroform anaesthesia in fish is *accompanied* by a marked fall in the oxygen intake, but there is, of course, nothing to show that this is the cause of the loss of irritability.

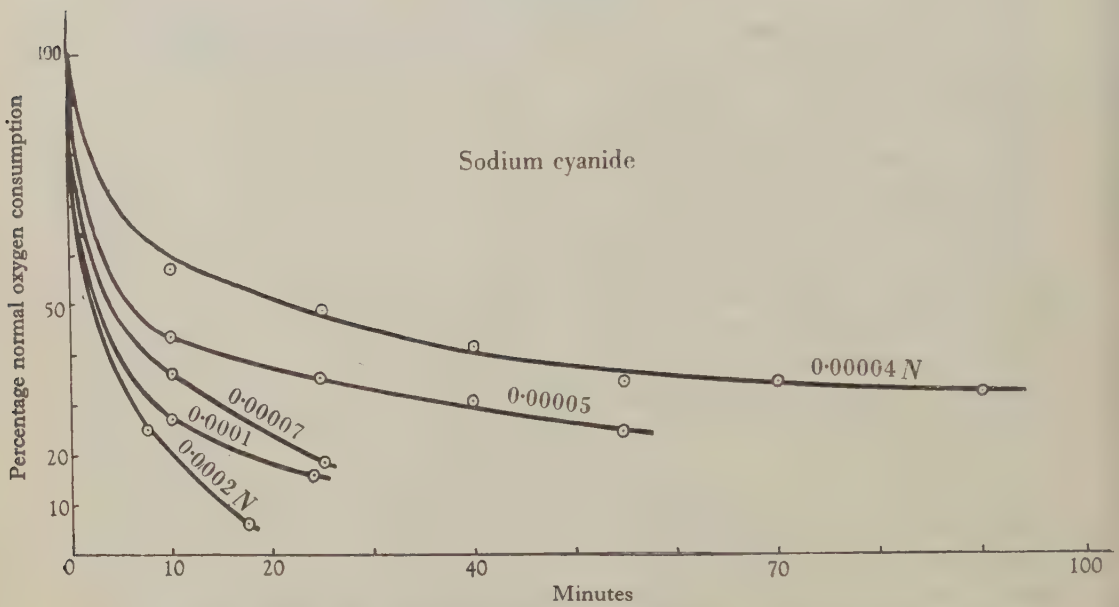

Fig. 3. Oxygen consumption curves for sodium cyanide solutions. In each experiment ten small fish were placed in the respiration flask. In the 0·0002 solution all the fish died in about 20 min.; at 0·0001 all died in about 28 min. The 0·00007 and 0·00005 experiments were discontinued when three fish ceased breathing; the fish in the 0·00004 solution were all alive when the experiment was discontinued in 100 min., one died subsequently in the aquarium. *p*H of all solutions adjusted to 7·0 with HCl.

SODIUM CYANIDE

The second respiratory depressant studied was sodium cyanide. The effect of hydrocyanic acid (and potassium cyanide, sodium cyanide and other salts forming HCN on solution) on respiration is well known; Krogh (1916, p. 68) reviews the older literature, Meldrum (1934) discusses its inhibitory effect on indophenol oxidase, catalase and peroxidase, a long bibliography of more recent literature is given by Commoner (1940), and Alexander, Southgate & Bassindale (1935) have investigated the toxicity of cyanide solutions to trout.

A selection of the large number of results obtained by the writer is given in Fig. 3. The critical concentration for *Gasterosteus* is about 0·00004 *N*, which depresses the respiration rate to 32% normal in about 90 min. Solutions of higher concentration are fatal, for when the oxygen intake is depressed below this level the respiratory

movements tend to stop more or less suddenly. In solutions of greater dilution the fish survive a considerable time; thus in 0·00003 *N* five fish survived 155 min., after this time had an oxygen intake of 55% normal and recovered rapidly on restoration to water.

In Fig. 4 a typical result is given recording the reactions of a single fish in a 0·00005 *N* solution. It was found possible to count the rate of opercular movement

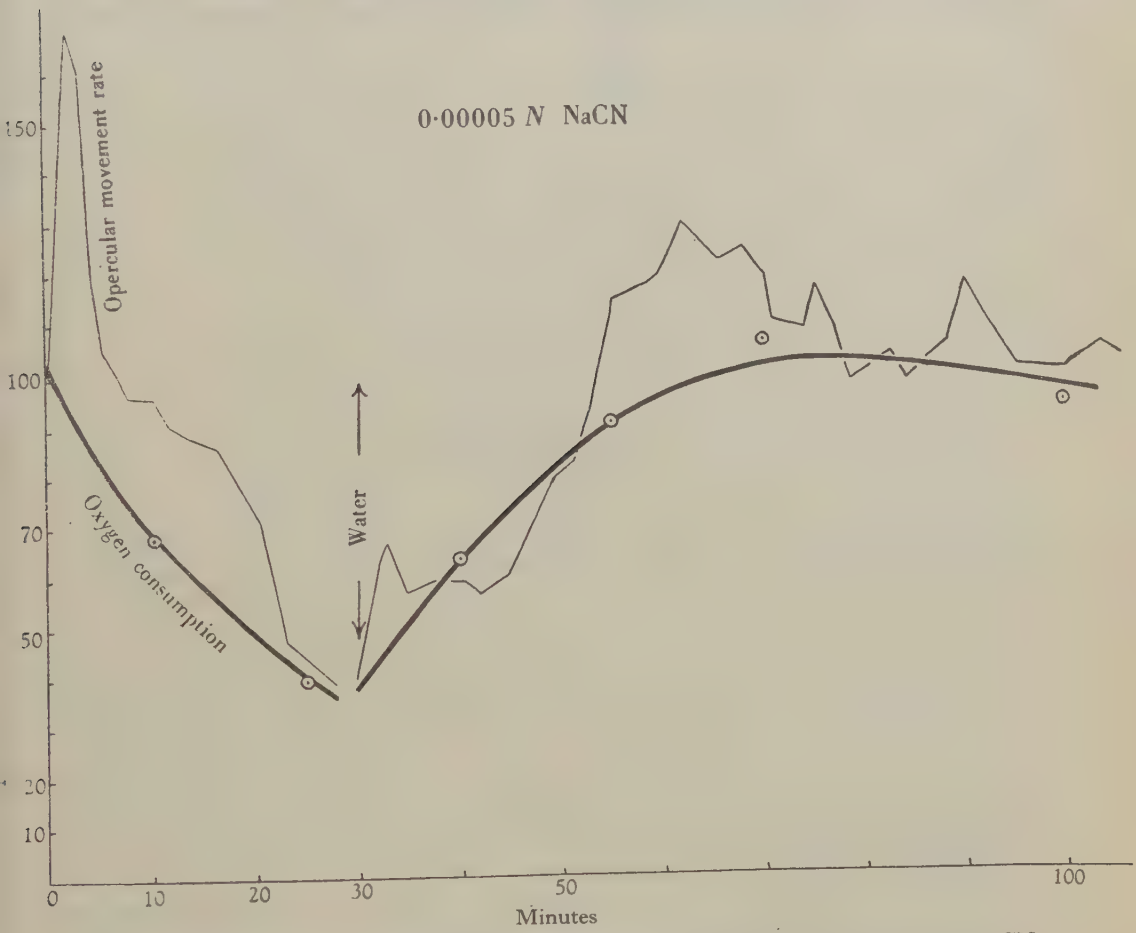

Fig. 4. Oxygen consumption and opercular movement rate curves for a single fish in 0·00005 NaCN, showing the recovery following the replacement of the cyanide solution by water at 30 min.

every 2 or 3 min.; it will be seen that on the introduction of the toxic solution the opercular movement rate rises temporarily and then declines very roughly in step with the oxygen consumption. On removing the cyanide solution and allowing water to run through the respiration flask (in 30 min.), both oxygen consumption and opercular movement rate return to normal in about 30 min. Recovery is therefore not very much less rapid than the recovery of fish after treatment with deoxygenated water (see Fig. 5).

The recovery power of fish after cyanide treatment is remarkable. Fish that have practically ceased breathing and lie helpless on their sides, on transference to well-aerated water soon begin energetic respiratory movements, in 15–20 min. regain their sense of balance, swim actively, and in 1 or 2 hr. appear perfectly normal.

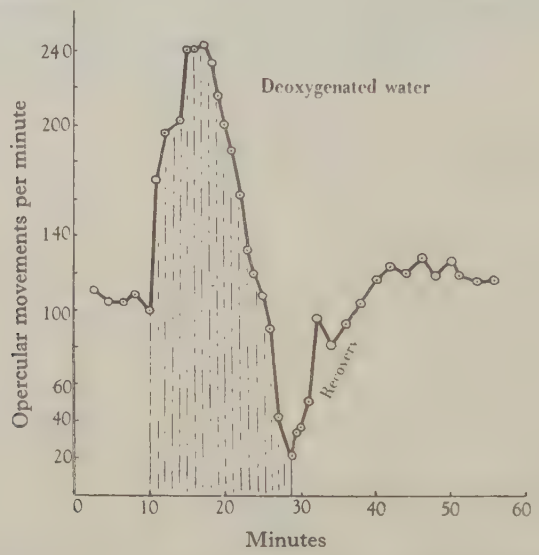

Fig. 5. Opercular movement rate graph for *Gasterosteus* in deoxygenated water. At 10 min. water boiled for several hours and cooled under paraffin was run into the respiration chamber. At 28 min. the supply of aerated water was restored (compare Ellis, 1937, p. 377).

SODIUM SULPHIDE

In dilute solution sodium sulphide is practically completely hydrolysed to form hydrogen sulphide and sodium hydroxide. The high toxicity of sulphides to vertebrates is well known, and it is generally accepted that its action on respiratory mechanisms closely resembles that of hydrocyanic acid. Sulphides are important pollutants, for they are formed by the decomposition of sewage and occur in the effluents from sugar-beet factories. The toxicity of sulphides to trout has been investigated by Longwell & Pentelow (1935), who state that the critical concentration for neutral solutions is approximately 1 pt. S/10^6 water, which causes the fish to 'overturn' in 25 min.

The study of the effect of sulphide solutions on the respiration rate of fish presents three difficulties: the solutions tend to be alkaline, in solutions of great dilution the H_2S is slowly oxidized to water and sulphur, and thirdly the H_2S reacts with iodine so that the normal procedure of the Winkler method is impossible. These difficulties were overcome by preparing a fresh solution for every measurement of the respiration rate (every 15, 12 or 10 min. as the case might be), adding sufficient sulphuric acid to make the solution neutral, and adding to each solution sample exactly sufficient iodine solution to neutralize the sulphide before adding the manganese chloride and other reagents of the Winkler method.

An example will help to make this clear. In an experiment with 0·0002 *N* Na_2S the schedule of operations was as follows:

Time	Procedure
Preliminary	Prepare large supply of tap water approximately air saturated at 17° C. Check concentration of stock sulphide solution by titration with standard iodine. Place fish in respiration flask and estimate oxygen consumption in water. Prepare 0·0002 solution by making up 1 ml. 0·5 *N* Na_2S to 2500 ml. Bring to *p*H 7 by adding 0·4 ml. *N*/10 H_2SO_4. Pour into supply aspirator.
0 min.	Set solution running through respiration flask.
2 min.	Fill sample bottle with outflowing solution, stopper, shake off excess, empty into beaker, titrate with *N*/100 iodine. Note volume of iodine required, in this case 2·4 ml.
4 min.	Refill sample bottle from outflow. Add 2·4 ml. iodine, stopper with air excluded, shake.
5 min.	Stop solution flow. Add $MnCl_2$, etc., to sample, titrate with *N*/30 thiosulphate. This gives oxygen content of running solution.
12 min.	Prepare fresh 2500 ml. of sulphide solution for second measurement of oxygen consumption.
15 min.	Collect water sample from respiration flask. Refill this with the fresh solution just prepared and allow this to run through rapidly. Add 2·4 ml. iodine to water sample as at 4 min. then Winkler reagents and titrate. The difference in the thiosulphate readings for the samples at 5 and 15 min. is a measure of the oxygen consumed by the fish in that period.
19 min.	Fill sample bottle from outflow to check oxygen content of fresh solution.
20 min.	Stop solution flow. Measure oxygen content of sample just collected. Prepare solution for 35–45 min. time period.
30 min.	Collect sample from respiration flask as at 15 min. etc. Set fresh solution running et seq.

At first it did not prove possible to carry out all these operations in the time available. A considerable improvement was effected by having the stock sulphide solution, manganous chloride and alkaline iodide solutions and hydrochloric acid delivered from long-nozzled burettes, and using the iodine and thiosulphate solutions in micro-burettes with self-filling arrangements. After some practice the respiration rate could be measured every 10 min., but in most experiments the 15 min. interval was retained.

A selection of the curves obtained with the sulphide solutions is given in Fig. 6, and it will be seen that the general result closely resembles that observed with cyanide. The sulphide solutions, however, are somewhat less toxic and at great dilution the depression of the respiration rate comes on slowly. Thus a 0·0002 *N* Na_2S solution depresses the oxygen intake to about 33% normal in 90 min. like a 0·00004 cyanide solution, but the sulphide curve does not have the pronounced downward sweep of the cyanide curve and descends gently. 0·0002 *N* is about the critical concentration for sodium sulphide, at greater dilution the survival time lengthens with great rapidity.

It proved impossible to measure the respiration rate of a fish and also make periodic counts of its rate of opercular movement. In Fig. 7 one curve records the fall in oxygen consumption of a fish in a 0·00035 *N* solution and the other records the opercular movement rate of another fish which, in a separate experiment, died in approximately the same time as the first. The result resembles closely that depicted in Fig. 4.

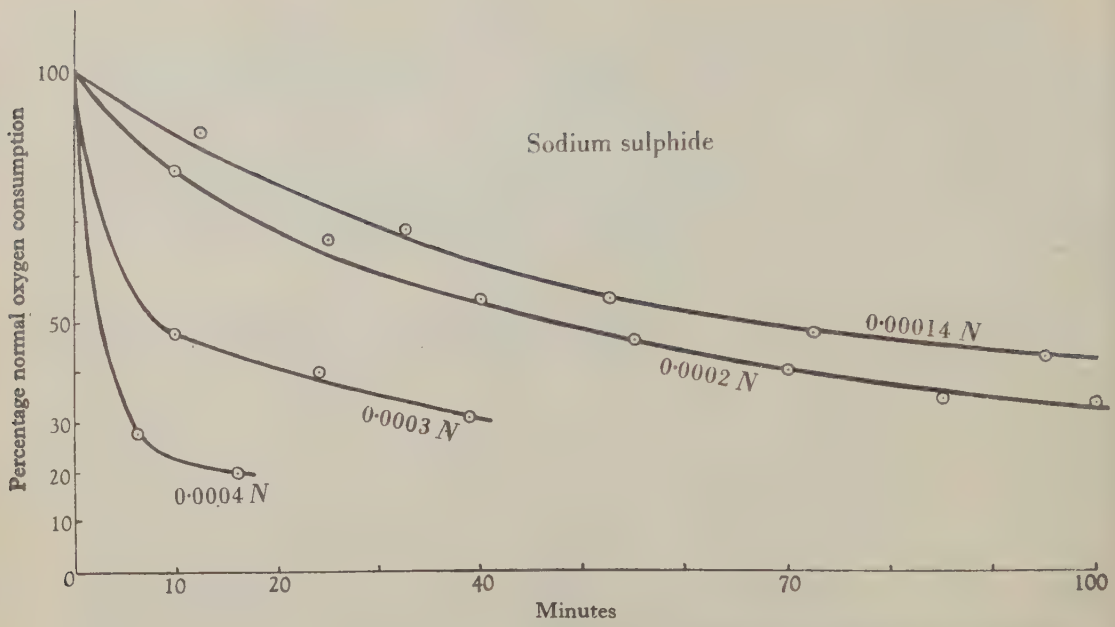

Fig. 6. Oxygen consumption curves for sodium sulphide solutions. In each experiment three fish were placed in the respiration flask. At the three higher concentrations the experiment was discontinued when one of the fish died; at 0·00014 *N* all were alive when the experiment was discontinued in 100 min. and all recovered completely on being returned to the aquarium.

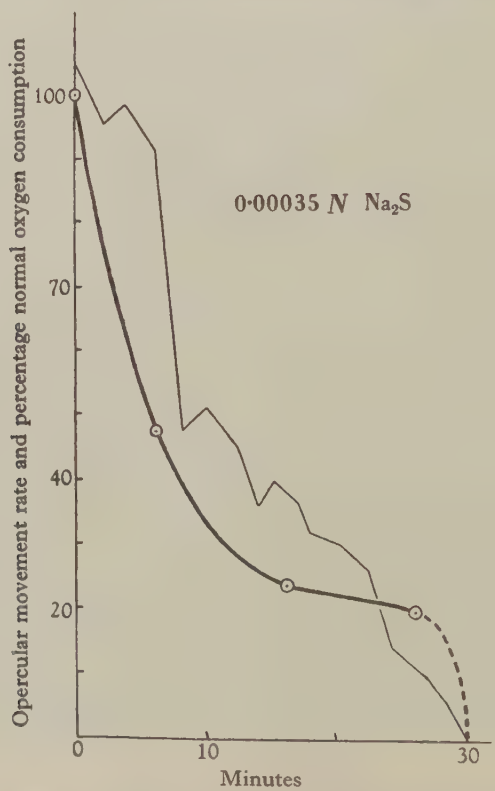

Fig 7. Experiment with a single fish in 0·00035 *N* Na_2S showing how the rate of opercular movement falls with the rate of oxygen intake.

Comparatively concentrated sulphide solutions are rapidly fatal. A fish placed in a 0·001 *N* solution loses its sense of balance almost at once and ceases breathing in about 6 min. If fish are removed from sulphide solutions before they cease making respiratory movements they exhibit the same remarkable power of recovery observed in the case of cyanide.

HEAVY METAL SALTS

The first heavy metal salt studied was mercuric chloride, and a number of oxygen consumption curves were plotted for solutions ranging in concentration from 0·0003 to 0·00004 *N*, covering the survival time range 30–110 min. The two curves in Fig. 8

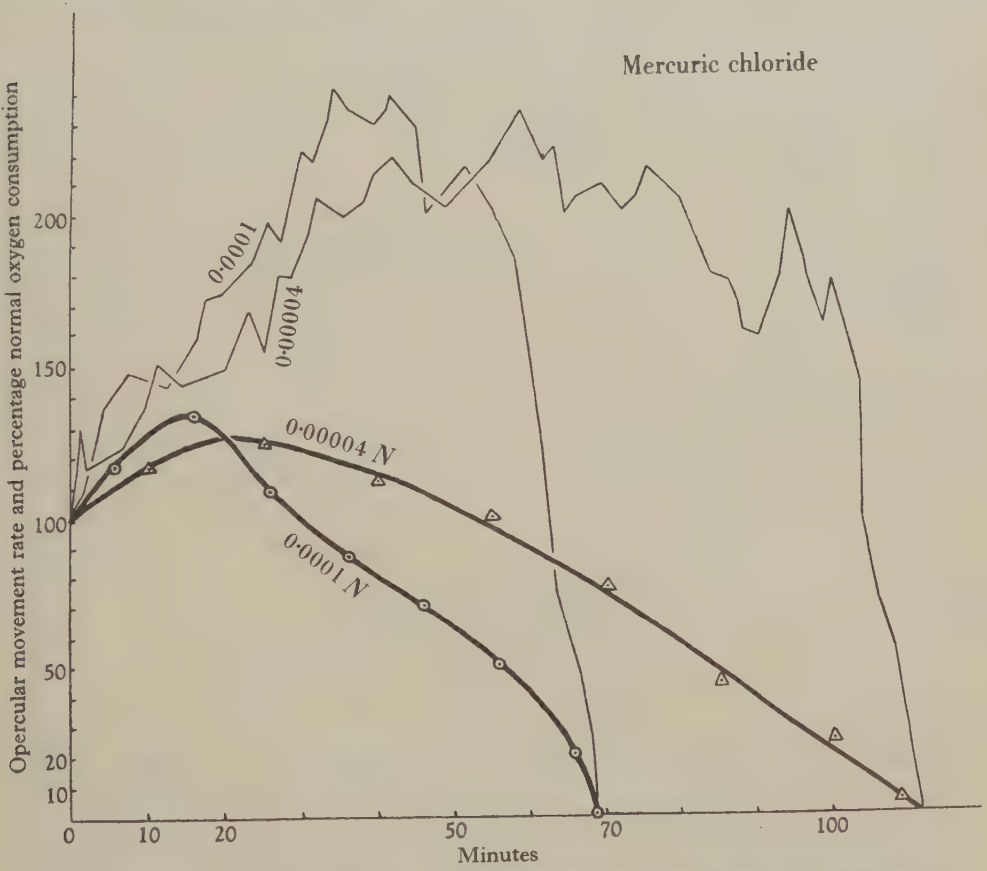

Fig. 8. Oxygen consumption (heavy), and opercular movement rate (light) curves for mercuric chloride solutions; a single fish at each concentration.

are typical results. It will be seen that following the introduction of the toxic solution the oxygen consumption of the fish rises, rapidly attains a maximum value which in different experiments varied from 120 to 152% normal, and then steadily declines until the fish dies. This rise in oxygen consumption is probably related to the increased activity the fish displays on the introduction of the solution (compare the

writer's results (1941) with *Polycelis* and *Gammarus*), and is accompanied by a marked increase in the rate of ventilation which persists after the rate of oxygen intake is well on the decline. Thus in the case of the 0·00004 *N* solution the opercular movement rate continues to rise over the time period 20–70 min. while the oxygen intake declines; then the fish begins to become exhausted, the ventilation rate drops,

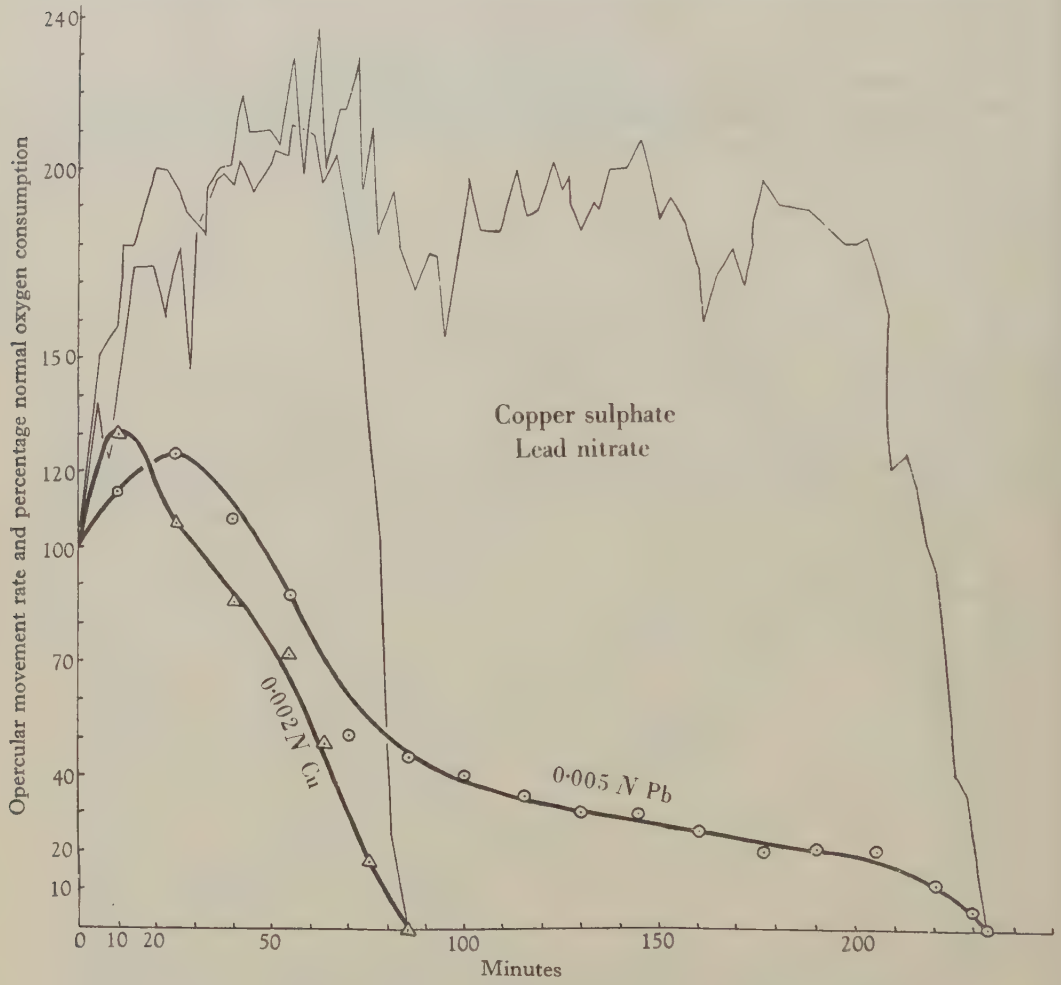

Fig. 9. Typical results for copper sulphate and lead nitrate. Other details as Fig. 8.

at first hesitatingly and then precipitately. The 0·0001 *N* curves are generally similar, the process being compressed in time.

A number of similar results were obtained with other heavy metal salts. Two typical results are given in Fig. 9, for 0·002 *N* $CuSO_4$ and 0·005 *N* $Pb(NO_3)_2$. The curve for lead nitrate is interesting in that the fish persisted in rapid and energetic breathing for over an hour after its oxygen intake had been reduced to less than 30% normal. This is unusual; in ten separate experiments with mercuric chloride the

average value for the oxygen intake at the time the rate of opercular movement began its final precipitate descent was 37% normal. This, it will be noticed, is not greatly different from the critical level for oxygen intake in the case of NaCN and Na_2S.

The general interpretation of the results is fairly obvious. In the case of NaCN and Na_2S gaseous interchange at the gill surfaces is not interfered with; less oxygen is taken in because the tissues cannot utilize it, less carbon dioxide is produced. It appears to be generally accepted that in the higher animals an increase in the carbon dioxide content of the blood is the chief stimulant to increased speed and amplitude of the respiratory movements. The decline in carbon dioxide production is therefore followed by a decline in the ventilation rate, the respiratory organs do not strive to

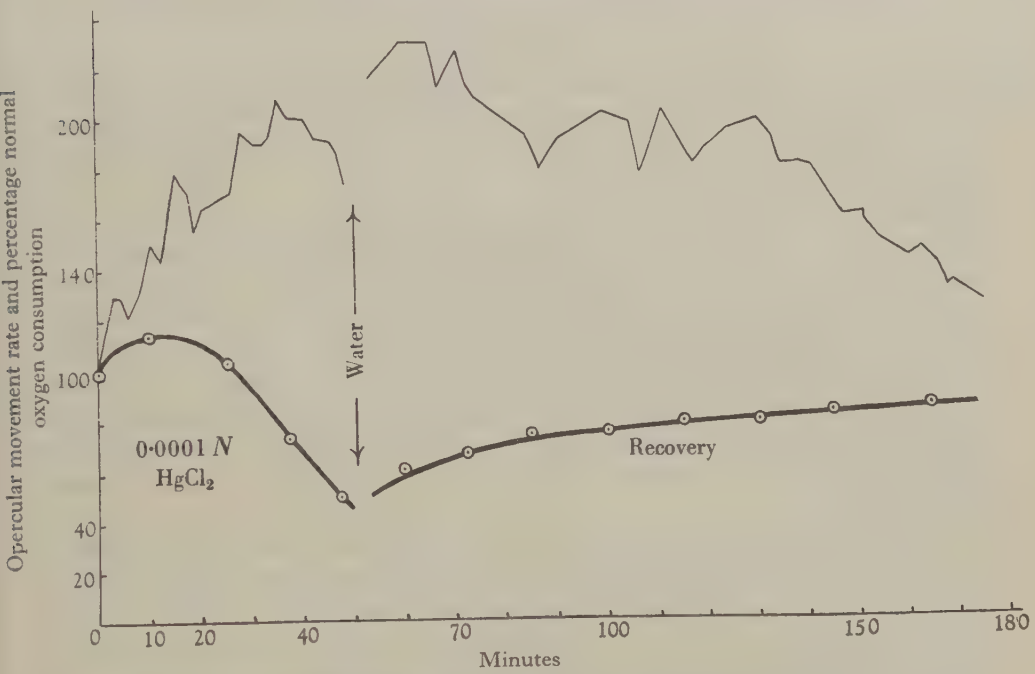

Fig. 10. Experiment showing the slow recovery of a fish after 50 min. exposure to 0·0001 $HgCl_2$.

supply more oxygen than the tissues can utilize. On the removal of the poison the tissues gradually recover their power of utilizing oxygen, more carbon dioxide is produced, the respiratory centre is stimulated and the ventilation rate rises.

In the case of a fish in a heavy metal salt solution, on the other hand, respiration is obstructed at the gill surfaces; the combined effect of oxygen deprivation and carbon dioxide retention results in an increase in the rate and depth of breathing. A vicious circle is set up, the fish is soon breathing at the maximum rate of which it is capable, but with continuing fall in the oxygen intake the animal becomes exhausted; the ventilation rate cannot be maintained and death results.

Recovery on removal from the solution after this asphyxiation process is far advanced, is slow and uncertain. Fig. 10 is a typical result showing the recovery of a

fish when the solution was replaced at 50 min. with well-aerated tap water. The oxygen intake begins a slow upward climb and 2 hr. later does not exceed 80%. The experiment was discontinued at this point and the fish placed in an aquarium. Next day it appeared to have recovered completely. In other experiments of the same type cases were noted of fish making considerable progress towards recovery and then suddenly succumbing from exhaustion.

The writer hopes to extend this investigation. The eel, on account of its well-known ability to withstand oxygen deprivation, should make interesting comparison with the stickleback, and there are many more substances which are important stream pollutants and whose effect on the respiration of fish is unknown.

SUMMARY

A simple apparatus is described with which the oxygen consumption of a small fish may be estimated, first in water and then at successive time intervals in a toxic solution, so that the progressive effect of the solution on the rate of oxygen intake may be graphed.

Chloroform solutions of concentration 1/3000 to 1/5000 gradually depress the respiration rate and are ultimately fatal. A 14/100,000 solution appears to produce a state of anaesthesia in which the oxygen intake is about 30% normal.

Sodium cyanide solutions bring about a progressive decline in the oxygen intake which is closely accompanied by a decline in the rate of opercular movement. The critical concentration is about 0·00004 N, which depresses the respiration rate to 32% normal in 90 min. At greater dilution the survival time lengthens rapidly. Sodium sulphide solutions give very similar results but are somewhat less toxic than cyanide solutions, and the value for the critical concentrations is about 0·0002 N.

Heavy metal salts (mercuric chloride, copper sulphate, lead nitrate) produce at first an increase in the respiration rate. Then the oxygen intake declines, but the rate of opercular movement continues to increase, reaches 180–240/min., continues at this increased rate for some time in the case of dilute solutions and then falls rapidly when the oxygen intake is reduced to 38% normal.

It is concluded that in the case of cyanides and sulphides, where respiration is inhibited at the tissues, the close agreement between the oxygen intake and opercular movement rate may be attributed to the decline in carbon dioxide production. In the case of the heavy metal salts, on the other hand, where respiration is obstructed at the gill surfaces, it would appear that the carbon dioxide content of the blood is raised so that the respiratory centre is stimulated and the opercular movements increase in speed and amplitude. The continued fall in oxygen intake and accumulation of carbon dioxide maintains this effect for some time, but eventually the fish becomes exhausted, the respiratory movements fail and the fish dies.

The work concludes with some suggestions for further investigation.

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STUDIES ON STERILITY AND PRENATAL MORTALITY IN WILD RABBITS

I. THE RELIABILITY OF ESTIMATES OF PRENATAL MORTALITY BASED ON COUNTS OF CORPORA LUTEA, IMPLANTATION SITES AND EMBRYOS

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(With Four Text-figures)

INTRODUCTION

Sterility in a female mammal may result from failure to produce fertilized ova, or from subsequent death of the embryos. Although only the latter falls strictly under the heading of prenatal mortality, both are included in these studies. It has been shown already (Brambell, 1942, 1944) that in the wild rabbit by far the greater part of the wastage of ova is due to the death of the embryos at some stage after fertilization. This may be due in part to the fact that in the rabbit ovulation is not spontaneous, the animal remaining in persistent oestrus until copulation occurs or the breeding season ends. Once copulation occurs in an oestrous rabbit ovulation ensues approximately 12 hr. thereafter. Since very few oestrous rabbits fail to produce ova the proportion that do not become pregnant is small. Female rabbits that are not in oestrus, unlike the majority of other mammals, will copulate freely (Brambell, 1944), not only during pregnancy and pseudopregnancy, but even during the non-breeding season.

Although these special considerations apply only to the rabbit and a few other species, it appears that even in species that ovulate spontaneously, ovulation and fertilization rarely fail to occur at each oestrus, at least in the wild state during the effective breeding season, and that sterility, when it occurs, is due mainly to the subsequent death of the embryos. It seems probable that much of the sterility in farm-stock, and even in man, that is commonly attributed to failure to achieve fertilization, likewise is due, in fact, to the death and reabsorption or abortion of the embryos. It is only when abortion of relatively advanced embryos occurs that prenatal mortality can be recognized readily in the living animal. If the dead embryos are reabsorbed *in situ*, or if they are aborted at an early stage of development, the mortality is difficult or impossible to detect, except at autopsy. Moreover, if the mortality occurs early in development, before or soon after implantation, it may result in no interruption of the oestrous cycle, and then the fact that the animal was pregnant, not pseudopregnant, can be recognized only by dissection. The death of whole litters between the eleventh and fifteenth days of gestation, that has been shown to occur commonly in the wild rabbit (Brambell, 1942, 1944; Brambell &

Mills, 1944), and their subsequent reabsorption, results in the animals coming into oestrus again at or soon after the time when they would have done so had they been pseudopregnant; and the degree of development of the mammary glands at this time is comparable also. If this phenomenon occurred in tame rabbits the animals affected would be classed almost certainly as pseudopregnancies, unless the uteri were examined while reabsorption was proceeding.

It is apparent, from these considerations, that a thorough investigation of prenatal mortality, in at least one suitably selected species, is an essential preliminary to the study of sterility in mammals generally. Such an investigation should be on a large scale and should lead to the elaboration of suitable techniques, which could subsequently be adapted to other species. It should yield much information concerning the fundamental problems involved in the detection and analysis of prenatal mortality in mammals.

These studies have been undertaken with the objectives named above. They represent a continuation and extension of the preliminary work already reported (Brambell, 1942, 1944). They are based on extensive new data, but the earlier data have been reanalysed and are included also. The reason for their inclusion is that, in conjunction with the new data, they have yielded more information, rendering it possible to amplify and to correct the interpretations previously placed upon them.

The rabbit provides very favourable material for such studies because, in the first place, it can be investigated in the wild state under natural conditions and, at the same time, can be kept conveniently as an experimental animal; secondly, it is polytocous; thirdly, it has a short gestation period; and, fourthly, it is available in quantity and is cheap. Monotocous animals provide much more difficult material because, once the single embryo has died and has been removed, the mother is no longer pregnant. In such animals the mortality at early stages of development can be discovered only if examination takes place during the brief interval between the death and the removal of the embryos. At later stages the dead embryos are removed by abortion as a rule, and then the involution of the uterus is more rapid than if reabsorption had occurred, with a consequent decrease in the period during which the loss can be recognized at autopsy. Polytocous animals that lose the whole litter simultaneously present the same difficulty as monotocous species, in that they drop out of the category of pregnancies, but even so, in them the dead embryos often are reabsorbed *in situ* and the mortality is detectable at autopsy, unless the animal was near full-term. The death of some only of the embryos in a polytocous animal seldom terminates pregnancy as the dead embryos are reabsorbed without interfering seriously with the course of development of the remainder. In such cases, since the corpora lutea in the ovaries and the sites in the uteri, where the dead embryos were reabsorbed, are remarkably persistent structures, often remaining identifiable until parturition, as in the rabbit, the total mortality of ova throughout the period of pregnancy up to the time of examination can be determined from comparison of counts of the corpora lutea, implantation sites and embryos. At any given stage of pregnancy the discrepancy between the number of living embryos in the uteri and of corpora lutea in the ovaries, except for certain qualifications that will be considered in this paper, is equivalent to the total loss of ova from the time

of ovulation until the animal is examined. Further, once implantation has occurred, the implantation sites provide a means of distinguishing between the loss of ova that occurred before implantation and that which occurred after implantation. The excess in the number of corpora lutea over the number of implantation sites is equivalent to the number of ova, fertilized or unfertilized, lost before implantation in surviving litters. The excess in the number of implantation sites over the number of living embryos is equivalent to the loss after implantation.

Since all the subsequent statistical work is based on counts of corpora lutea, implantation sites and embryos it is necessary first to inquire into the accuracy of these. The newly ruptured follicle gradually develops into a corpus luteum, which attains its maximum size during mid-pregnancy. Thereafter it remains constant in size until it begins to regress rapidly a few days before parturition, at which time in the rabbit it is still quite large and easily visible to the naked eye. After parturition it regresses more rapidly and soon disappears. If there is any tendency to overlook corpora lutea when counting, it might be expected that the incidence of such errors would be greater at the beginning and end of pregnancy, and least at mid-pregnancy, during the period of maximum development.

Similarly, implantation sites undergo regression after the death of the embryos. They do so relatively rapidly in animals that have ceased to be pregnant. The rate of reabsorption of the embryos and regression of the sites in such animals, in which the death of all the embryos at known stages of development has been induced experimentally, will be the subject of another paper that is in preparation. It is possible, indeed probable, that the rate of regression is retarded in animals that, owing to the survival of some of the embryos, continue to be pregnant, but it is certainly necessary to investigate whether, in such cases, the sites of embryos that have died soon after implantation persist until full-term or whether counts made near the end of pregnancy are liable to error through the disappearance of such sites.

Finally, although counts of embryos after implantation can be relied on, since the embryos are unmistakable at that time and become increasingly conspicuous thereafter, counts made before implantation, because of the technique and the small size of the ova or early embryos, are liable to serious error, and it is desirable to know if they are of any significance. Before implantation the ova or early embryos can be recovered only either by the perfusion of the Fallopian tubes or uteri as the case may be, or by the much more laborious method of serially sectioning these organs. The labour involved in the latter process is prohibitive in large samples of an animal as big as the rabbit. Although the proportion of ova and unimplanted embryos that can be recovered by perfusion in rabbits is high, it is difficult for technical reasons that have been outlined previously (Brambell, 1942) to recover them all, and impossible to be certain of having done so in every case. Moreover, unfertilized ova are particularly difficult to recover once they have traversed the Fallopian tube and have entered the uterus. Probably unfertilized ova are quickly eliminated from the uterine tract and, when all the ova are unfertilized, their passage through the Fallopian tubes may be accelerated. Consequently the proportion of such ova recovered is likely to be substantially less than the true proportion.

The errors introduced into the counts of corpora lutea, implantation sites and embryos through these causes are dealt with in this paper. A section is devoted also to showing that the loss of ova before implantation is unconnected with that occurring after implantation. It is convenient to introduce this here, since the subsequent statistical work, dealing with these two fractions of the total prenatal loss respectively, falls naturally into two parts.

MATERIAL

The technique originally employed has been described in detail already (Brambell, 1942, 1944) and it is only necessary to mention here those respects in which it has been modified and improved. First, during the earlier work, the uteri of visibly pregnant animals were fixed whole and the embryos dissected out and examined subsequently. This method was laborious and was liable to result in distortion of small embryos by pressure resulting from contraction of the uterine wall during fixation. Consequently during 1943 and subsequent years the uteri of visibly pregnant animals, after removal, were pinned out in a dissecting dish, opened under saline, and examined under a binocular dissecting microscope before fixation. This procedure permitted of more critical examination of the embryos and embryonic membranes, with a consequent increase in precision in determining whether they were developing or were in process of reabsorption.

Secondly, during and after 1944, whole mounts were made of tubal and early uterine ova, recovered by transfusion, instead of serial sections. They were mounted direct from water in gum chloral containing borax-carmine. The gum chloral was made up on the usual formula:

Distilled water	50 c.c.	Glycerine	20 c.c.
Gum arabic	40 g.	Chloral hydrate	50 g.

One part of borax-carmine was added to four parts of gum-chloral. The stain was made up by dissolving 4 g. borax and 3 g. carmine in 100 c.c. of hot distilled water and filtering. Mounts made with this medium were permanent, and the ova were stained adequately for determining the stage of development after the mount had been in the slide-drying oven for a few hours.

Both these modifications of technique resulted in a substantial saving of time and enabled the total prenatal mortality in a sample to be determined within a few hours of having received it. Since both involved the use of fresh material, they did lengthen somewhat the routine to which a sample had to be subjected immediately it was received; a serious consideration when the usual sample of thirty to forty pregnant females was received late in the evening, involving several hours' work for two people.

The accumulation of data rendered it desirable to record them on perforated index-cards which could be sorted mechanically, so as to reduce the labour of analysis. The system adopted was the Cope-Chat Paramount sorting system, recommended to us by Dr C. Elton, of the Bureau of Animal Populations, Oxford, and it has proved entirely satisfactory. The card employed was designed in 1943, when the work had reached a sufficiently advanced stage to form a clear idea of what

was required. The design, which is reproduced in Fig. 1, has proved efficient and may be of use to others. All the data have been entered on these cards, without which analysis of so much material would have been so laborious as to be almost impracticable.

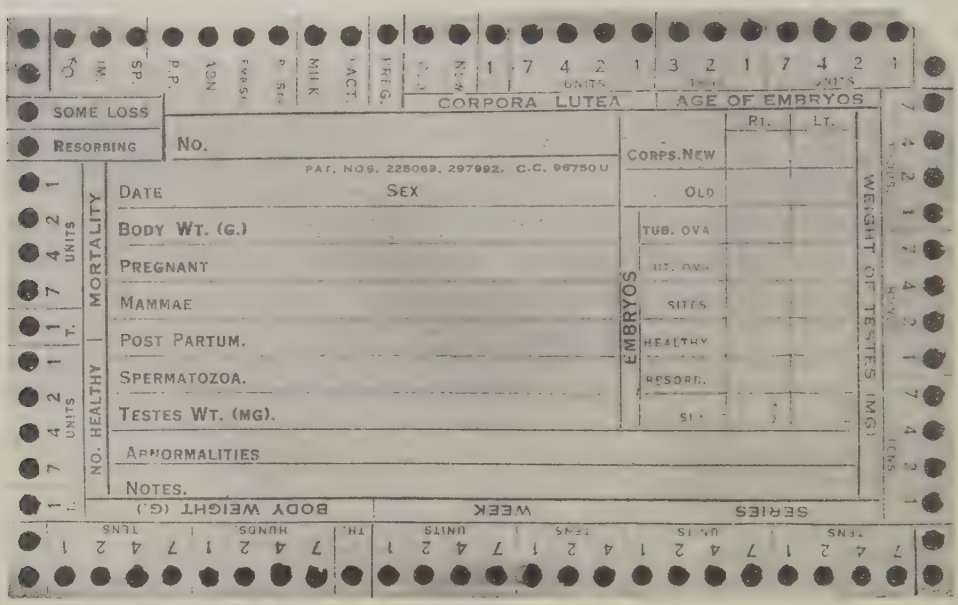

Fig. 1

The material consists of 7137 wild rabbits obtained between 7 February 1941 and 30 May 1945, all of which were examined, dissected and recorded. These form eleven series according to the year and locality. Males were only included in those series intended to provide information regarding the duration of the breeding season. One intersex and two hermaphrodites were obtained. The localities, dates and sex distribution of each series are given in Table 1.

Table 1. *Summary of material*

Series	Locality	Dates	♂♂	♀♀	⚥
01	North Caernarvonshire	7. ii. 41 to 16. iv. 41	174	181	—
0	South Caernarvonshire	23. iv. 41 to 30. xii. 41	686	655	1
1	South Caernarvonshire	6. i. 42 to 26. vi. 42	97	693	—
2	North Anglesey	4. ii. 43 to 18. iii. 43	1	207	—
3	South Anglesey	16. xi. 43 to 13. vi. 44	256	613	1
4	South Caernarvonshire	17. xi. 43 to 20. xii. 44	677	1247	1
5	Norfolk	15. ii. 44 to 31. iii. 44	—	229	—
6	Norfolk	15. ii. 44 to 31. iii. 44	—	273	—
7	Dumfries and Kirkcudbright	12. iv. 44 to 7. v. 44	—	187	—
8	Dumfries and Kirkcudbright	12. iv. 44 to 7. v. 44	—	305	—
9	South Caernarvonshire	3. i. 45 to 30. v. 45	154	499	—
		Total	2045	5089	3

Particulars of series 01, 0 and 1 have been published already. However, since the various series differ from each other in certain significant respects, such as the

type of country from which the rabbit came, the methods by which they were obtained, the size and frequency of the samples aimed at, and the special treatments to which some were subjected as the result of experience or to throw light on some particular aspect of the problem, the characteristics of each series are set down here for comparison, since they affect the purpose for which the data can be used legitimately.

Series 01 (specimens labelled R 1–355). Obtained on the Vaynol Estate, near Bangor, in north Caernarvonshire, consisting of a park, with woods, coverts and some arable land, near the sea. The majority were shot or caught by dogs, after flushing from cover or bolting with ferrets. They were received in good condition on the day they were killed or the following morning. A weekly sample of about forty rabbits of both sexes was aimed at but not always achieved.

Series 0 (specimens labelled R 356–1697). Obtained around Brynkir and on the Lleyn Peninsula in south Caernarvonshire except for a single sample of twenty-one rabbits on 23 October from the Vaynol Estate in north Caernarvonshire. The country is hilly, intersected by rivers and near the sea. It consists of farmland, mainly lowland grass, with stretches of rough ground, covered with bracken and gorse, and some patches of woodland and of bog. The fences are mainly old rotten banks, often with hedges. Near the sea there are areas of dunes. The whole area is very heavily infested with rabbits. Practically all the material was trapped by the trappers of the War Agricultural Committee. Some of it was in excellent condition when examined, but some was not and a few carcasses were so bad when received that they were useless. Weekly samples of forty rabbits of both sexes were desired, but the numbers obtained often fell below the target.

Series 1 (specimens labelled R 1698–2487). Obtained from the same locality and in the same way as series 0. Weekly samples of about forty rabbits of both sexes were continued until 3 February. Thereafter the collection of males was discontinued and a weekly sample of forty females was the target.

The uterine tracts of all females of series 01, 0 and 1 were preserved. The embryos in visible stages of pregnancy were not dissected out and examined until after fixation. In series 01, 0 and that part of series 1 collected during January and February, the mammary glands were classified simply according to whether or not they contained milk. Thereafter they were classified according to whether (*a*) they were definitely suckling, (*b*) they had milk in the glands but were not certainly suckling, or (*c*) they had no milk in the glands. Tubal and early uterine eggs were embedded and serially sectioned.

Series 2 (specimens labelled AR 1–208). Obtained in the northern part of the island of Anglesey, within a radius of 5 miles of Llanerchymedd. This is low-lying country consisting mainly of grassland, interspersed with stretches of bog and rough ground covered with bracken and gorse. Old rotten banks fence the small fields and the population of rabbits is heavy. All the rabbits were trapped and they were received in fairly good condition. The object was to obtain a small sample of female rabbits during the height of the breeding season to determine whether or not whole litters died and were reabsorbed after implantation. Weekly samples of up to forty rabbits were examined, until over 200 had been obtained. All the swellings in visible

stages of pregnancy were opened before fixation under saline and full notes made of dead and reabsorbing embryos and, wherever possible, the order in which they had died according to their position in the uterus recorded. No attempt was made to obtain by transfusion tubal or early uterine ova and hence 0–6-day stages of pregnancy were not identified.

Series 3. Obtained in south Anglesey within a radius of 5 miles of Capel-Mawr, near Trefdraeth. This is low-lying farmland, mainly consisting of grass, with some arable and rough land. It is divided up into small fields by banks and hedges. It adjoins extensive areas of marsh to the east and of dunes to the south and carries a heavy population of rabbits. The majority of the rabbits were trapped, but some were netted, and some ferreted. They were received in very good condition, many of them being still warm and few more than 12 hr. dead. A weekly sample of forty females was the target. The full routine of examination, described at the beginning of this section, was employed for this sample and for the succeeding five series obtained in 1944. Non-pregnant uteri and those from which early stages had been recovered by transfusion were not preserved as a rule, nor were the uteri and embryos of many of the very late stages. This omission, which applies equally to series 4–8, was unfortunate, for it prevented estimates of the proportion of whole litters lost after implantation being made subsequently from the proportions of recently pregnant uteri in which reabsorption or parturition had occurred. Such uteri can be distinguished histologically. This series was obtained at the same time as series 4 and the treatment was similar, hence the two are strictly comparable.

Series 4. Obtained from the same areas of south Caernarvonshire and in the same way as series 0 and 1. A weekly sample of forty females over a period of a year was the target. The treatment was the same as in series 3.

Series 5 *and* 6. Obtained in Norfolk within a radius of 15 miles of Norwich. The area was mainly grass and arable lowland, with banks and hedges bordering the fields. Some of the material came from Foxley Wood, of some 500 acres extent. Most of the animals were trapped but many were ferreted and shot. None of the animals was more than 24 hr. dead when received, and the majority less than 12 hr., so their condition was good. The treatment was the same as in series 3 and 4. The two series differ only in that in series 5 it was intended to secure a random sample of approximately thirty adult females per week, whereas series 6 consisted only of females presumed to be pregnant because of the presence of recent corpora lutea in the ovaries. The aim was to secure a sample of approximately 500 females as quickly as possible during the height of the breeding season, to determine the incidence of prenatal mortality and to compare it with that experienced in North Wales.

Series 7 *and* 8. Obtained in Dumfriesshire and Kirkcudbrightshire within 15 miles to the south and to the west of the town of Dumfries respectively. The country is hilly, frequently rising above 600 ft., consisting mainly of grass and arable land, with hedges bordering the fields. The animals were mostly trapped, but a few were shot. All were received in very good condition within a few hours of death. The treatment and aim was the same as in series 5 and 6, and the target was reached in a shorter time. Series 8, like series 6, consisted of animals selected because they were believed to be pregnant.

Series 9. Obtained in south Caernarvonshire from the same area and in the same way as series 0, 1 and 4. This sample was designed to supplement the information already obtained regarding the breeding season in Caernarvonshire, to provide material for embryological investigation, and to elucidate certain points regarding the incidence of prenatal mortality. Since it was necessary to keep some of the swellings in each visible pregnancy intact for subsequent sectioning, it was not possible to determine by dissection the full number of embryos that were dead and reabsorbing. A special point was made of preserving all non-pregnant and early pregnant uteri, so as to determine histologically which were post-partum and which post-reabsorption. Great care was taken also to recover, as far as possible, all tubal and uterine ova, so as to estimate the proportion of ova lost through not being fertilized.

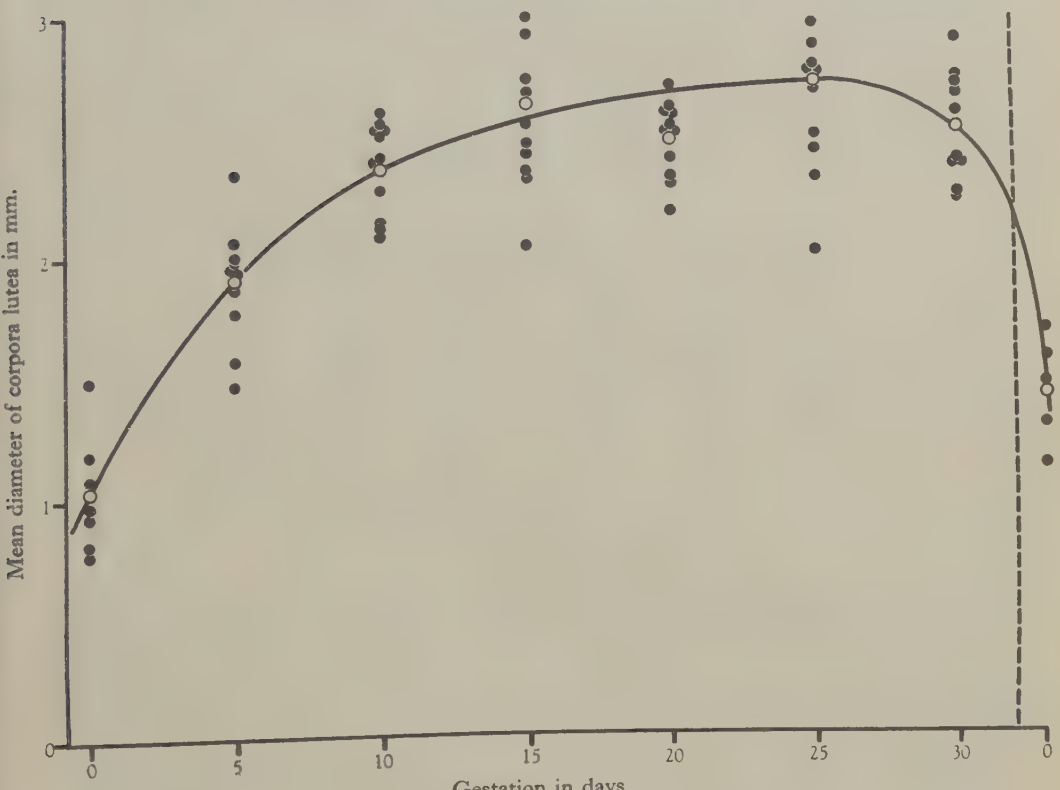

Fig. 2

The dotted line represents the time of parturition.

OBSERVATIONS

The experimental error of counts of corpora lutea

The corpora lutea in rabbits attain a size of 2·5–3·0 mm. in diameter during pregnancy. They are then comparatively large and conspicuous structures which can be easily identified and counted with the naked eye in fresh ovaries. They develop slowly and only attain their maximum size at mid-pregnancy, as can be seen from the growth curve given in Fig. 2 and the data of the mean size of the corpora lutea in each

of seventy-four pairs of ovaries given in Table 2. Thereafter they do not decrease in size appreciably until the last 2 or 3 days of gestation, and they are still easily visible at the time of parturition. They retrogress rapidly after parturition, or during the later stages of the reabsorption of a litter that has died *in utero*, and then they soon cease to be countable. Even the newly ruptured follicles and the corpora lutea in early stages of development appear to be easy to count macroscopically, especially as the rupture point is marked by a small pimple on the surface of the ovary at this time. In practice difficulty is experienced occasionally when the rupture point of a young corpus luteum is close to the mesovarium, where it may be overlooked unless the Fallopian tube is completely reflected so as to expose the whole surface of the ovary or when two fully formed corpora lutea have developed so close together as to appear to be fused. Sometimes the corpora lutea are difficult to count in the ovaries of a young animal which has recently attained puberty and ovulated for the first time, probably because the ovaries are so translucent at this time that

Table 2. *Mean diameters (in mm.) of corpora lutea in pairs of ovaries at successive stages of pregnancy*

0 day	5 day	10 day	15 day	20 day	25 day	30 day	1 day post-partum
0·78	1·48	2·10	2·06	2·20	2·02	2·25	1·12
0·82	1·58	2·13	2·35	2·32	2·33	2·26	1·29
0·94	1·78	2·15	2·37	2·34	2·45	2·39	1·38
0·98	1·88	2·29	2·45	2·42	2·51	2·39	1·57
1·09	1·93	2·40	2·49	2·53	2·71	2·40	1·68
1·19	1·95	2·42	2·57	2·54	2·78	2·60	
1·50	2·00	2·52	2·70	2·55	2·79	2·68	
	2·00	2·53	2·75	2·61	2·80	2·72	
	2·07	2·55	2·94	2·62	2·89	2·75	
	2·35	2·56	3·01	2·63	2·98	2·91	
		2·61		2·72			
1·04	1·92	2·38	2·57	2·50	2·63	2·53	1·41

degenerating follicles within the ovaries appear as opaque masses which can be confused with developing corpora lutea. Sometimes one or more abnormally small corpora lutea are present in a pair of ovaries together with several larger ones which are all subequal in size and which they resemble in colour and texture. It is then difficult to decide whether these small ones are corpora lutea vera, which should be counted with the others, or whether they are corpora lutea atretica formed from unruptured and immature follicles, which should not be counted. The decision in such cases is necessarily somewhat arbitrary, unless the rupture points happen to be clearly visible.

The practice adopted was to count carefully the corpora lutea in the ovaries first, then to count the uterine swellings, if these had developed, or to dissect out the tract and transfuse out early stages. If the number of embryos in either tube or uterus exceeded the number of corpora lutea in the corresponding ovary, the luteal count was very carefully checked. If, as was common, the discrepancy still could not be resolved, the ovaries were fixed, subsequently cut with a safety-razor blade into

freehand sections about 1 mm. thick, and the corpora lutea counted under the dissecting microscope. All corpora lutea more than a day old could be identified readily by this method, so that it was found to be unnecessary to imbed and make microtome sections of the ovaries. Some of the discrepancies were resolved by this means but others were found to be real.

Although the macroscopic counts of corpora lutea made at the time of dissection appear to the observer to admit of a high degree of precision, notwithstanding the occasional difficulties experienced, which are enumerated above, it is desirable to know the magnitude of the experimental error. This is necessary if reliance is to be placed on estimates of prenatal mortality based on counts of corpora lutea. If it is supposed that the precision of identification of corpora lutea depends on their size then it follows that the risk of omitting some from the counts would be greater before they have attained their full development and after they have begun to regress. To test this hypothesis all the data of the size distribution of the sets of corpora lutea in pregnant animals, grouped according to the stage of pregnancy, have been examined and are given in Table 3, together with the mean size of litter and its standard error in each group. The differences of the means and the standard errors of the differences are:

Groups 0– 5 days and 6–10 days	$0{\cdot}197 \pm 0{\cdot}094$
Groups 16–20 days and 21–25 days	$0{\cdot}184 \pm 0{\cdot}104$
Groups 21–25 days and 26–32 days	$0{\cdot}221 \pm 0{\cdot}102$

The first and the last of these are significant, the difference being more than twice its standard error in each case, while the second is scarcely significant. The maximum difference, between the means of groups 16–20 and 26–32, is $0{\cdot}405 \pm 0{\cdot}093$, and is thus more than four times its standard error. Regarding Table 3, it is difficult to

Table 3. *Distribution of number of corpora lutea in a set according to stage of pregnancy*

No. of corpora lutea	Stage of pregnancy in days					
	0–5	6–10	11–15	16–20	21–25	26–32
13	1	—	—	—	—	—
12	—	1	—	—	—	—
11	—	—	—	—	1	1
10	1	4	3	2	1	4
9	15	14	19	16	8	8
8	45	50	51	30	29	32
7	102	100	95	99	64	68
6	157	119	135	129	84	129
5	130	101	123	92	83	114
4	88	77	74	53	58	116
3	38	19	27	19	17	26
2	4	1	1	—	—	—
1	—	2	—	—	—	—
Total	581	488	528	440	345	498
Mean	$5{\cdot}676 \pm 0{\cdot}062$	$5{\cdot}873 \pm 0{\cdot}070$	$5{\cdot}830 \pm 0{\cdot}065$	$5{\cdot}909 \pm 0{\cdot}067$	$5{\cdot}725 \pm 0{\cdot}079$	$5{\cdot}504 \pm 0{\cdot}065$

account for the mean number of corpora lutea in a set being less at the beginning and end of gestatien than in mid-pregnancy other than by errors of omission in the

counts. It will be seen that the maximum difference between the means represents a discrepancy of 6·8% of corpora lutea counted. Since the error must be due in the main to the omission of corpora lutea from the counts, rather than to overcounts, it follows that estimates of the prenatal loss will be underestimates by the amount of the error.

The macroscopic counts of 180 pairs of ovaries were checked by microscopic counts made from freehand sections, to determine directly the error in the original counts. The ovaries in which the counts were to be checked were all derived from the Caernarvonshire material and the majority from series 01, 0 and 1. They were selected only as regards age, so that thirty pairs of ovaries were checked from each age group. Ovaries in which the counts had been checked previously because the number of embryos in a uterus appeared to exceed the number of corpora lutea in the corresponding ovary were excluded. The results are given in Table 4, in which

Table 4. *Estimate of error in macroscopic counts of corpora lutea*

Divergence of macroscopic from microscopic count	No. of litters counted microscopically (days)						
	0–5	6–10	11–15	16–20	21–25	26–32	Total
+3	1	—	—	—	—	—	1
+2	—	—	—	—	—	—	—
+1	2	2	3	—	—	—	7
0	21	24	21	23	21	24	134
−1	2	2	4	5	8	6	27
−2	1	1	2	2	1	—	7
−3	2	—	—	—	—	—	2
−4	—	1	—	—	—	—	1
−5	—	—	—	—	—	—	—
−6	1	—	—	—	—	—	1
No. of litters counted	30	30	30	30	30	30	180
No. of incorrect counts	9	6	9	7	9	6	46
% of incorrect counts	30	20	30	23	30	20	26·6
No. of corpora lutea counted	181	172	179	179	182	160	1053
% error	11·6	5·8	6·1	5·0	5·5	3·7	6·4
% deficit in macroscopic count	6·1	3·5	2·8	5·0	5·5	3·7	4·5

the divergence of the original macroscopic count from the microscopic check count is shown. The microscopic counts were made with great care and there was little probability of any corpora lutea having been overlooked. Sometimes an arbitrary decision had to be made, as in the macroscopic counts, as to whether a corpus luteum should be included in the count or classed as a corpus luteum atreticum and excluded; in most cases the corpora lutea atretica could be readily identified by their much smaller size and by the frequent presence of a central cavity, sometimes containing a blood clot. It will be seen that the original counts diverged from the check counts by 6·4% of corpora lutea, affecting 26·6% of litters. Instances of the original counts exceeding the check counts were much fewer than errors of omission in the original counts and were confined to the first half of the gestation period. The net error was a deficiency of 4·5% of corpora lutea in the original counts which is of the order required to account for the differences between the mean numbers of corpora lutea in a set in each age group, as shown in Table 3. This error, however,

was distributed throughout all the age groups and not confined to the beginning and end of gestation, as would be required to compensate for the smaller mean numbers of corpora lutea at these periods as given in Table 3, but the sample of thirty pairs of ovaries in each age group checked is too small to attach statistical significance to the age distribution.

Corpora lutea atretica. It is well known that corpora lutea atretica are formed from unruptured follicles during pregnancy in the ovaries of several mammals, including the baboon (Zuckerman & Parkes, 1932), the bank-vole (Brambell & Rowlands, 1936) and the mare (Cole, Howell & Hart, 1931). (For full discussion of the relevant literature see Brambell, in press.) These are smaller than the functional corpora lutea, they have no rupture points, since they are formed from unruptured follicles, and remains of the follicular antrum often persist as a cavity in the centre. Abnormally small corpora lutea, clearly differing in size from the other corpora lutea of pregnancy in the same pair of ovaries, were observed in some of the rabbits and were taken to be corpora lutea atretica. They were found to be present in twenty-nine of the 180 pairs of ovaries examined microscopically. There were thirty-three of these small corpora lutea, two being present in each of four pairs of ovaries, thus amounting to 1·7% of all corpora lutea in the 180 pairs of ovaries examined. They were at least as numerous in the first half, as they were during the second half, of pregnancy, and their histological appearance closely resembled that of the functional corpora lutea of pregnancy. These facts suggest that they are formed only at the beginning of pregnancy at the same time as the normal corpora lutea. Many had a central cavity containing some blood, but the thickened luteanized walls distinguished them from blood follicles. Some were situated so deep in the cortex that they must have originated from follicles which could not have ruptured. In most cases the number of embryos present was equal to or less than the number of normal-sized corpora lutea, but in four instances the number of embryos was greater, suggesting that in these at least the small corpora lutea had been formed from follicles that had ruptured and liberated functional ova. The majority of these small corpora lutea fell within the size limits of 1·4–2·0 mm. in diameter and therefore corresponded in size with mature follicles about to ovulate.

It may be concluded from these observations that corpora lutea atretica are formed in the wild rabbit at the time of ovulation from not more than 2% of the mature follicles, which have failed to rupture, and that none are formed subsequently during pregnancy. These corpora lutea can be distinguished as a rule by their small size, although, very occasionally, such abnormally small corpora lutea may be formed from ruptured follicles.

It has been shown by Heape (1905) that maturing follicles which undergo degeneration frequently give rise to blood follicles through rupture of the thecal blood vessels and extravasation of blood into the antrum. Only maturing follicles appear to degenerate in this way. The maturing follicles in oestrous rabbits that are prevented from mating, and which therefore do not undergo the final rapid growth that occurs in the 12 hr. interval between copulation and ovulation, degenerate after a few days and are replaced by others, as has been shown by Hill & White (1933) and

confirmed by Smelser, Walton & Whetham (1934). Many of these, according to Heape (1905), Hammond (1925) and Hill & White (1933), give rise to blood follicles, which are relatively persistent structures that are gradually absorbed in a similar manner to a blood blister in the skin. Consequently such blood follicles are present as a rule in the ovaries of unmated oestrous rabbits. The corpora lutea atretica referred to above, appear to arise, on the other hand, from maturing follicles which have undergone the final growth that occurs after copulation but which have failed to ovulate. They have then undergone the luteal transformation at the same time as the ruptured follicles of the same generation, but their affinity to blood follicles is shown frequently by the presence of some blood in the persistent remains of the undischarged antrum. True blood follicles do occur in the ovaries of wild rabbits, but they are not nearly so common as in tame rabbits, presumably because in the wild state rabbits copulate very soon after they come into oestrus. This suggests that blood follicles arise mainly, if not exclusively, from maturing follicles which retrogress before the time of ovulation.

Polyovuly. It has been shown (Brambell, 1944) that in the wild rabbit the numbers of embryos or implantation sites in the uteri may sometimes exceed the number of corpora lutea in the corresponding pair of ovaries. This could be due either to a single ovum having produced two or more embryos or else to a single follicle having liberated two or more ova; that is, either to polyembryony or polyovuly. Polyembryony in mammals results in the identical twins having a common chorion, presumably owing to the persistence of the zona pellucida during cleavage preventing complete separation of the blastomeres. Since no such synchorionic twins have been encountered in the rabbits examined, it appears certain that polyembryony cannot be the explanation of the discrepancy, which therefore must be due to polyovuly. This phenomenon will be apparent from the counts whenever it occurs in a litter that suffers no loss before implantation. It will be masked, however, in litters which suffer pre-implantation loss, since the resulting increase in the numbers of embryos will tend to compensate for the disappearance of others. For example, a litter which has suffered both the loss of one embryo and the production of two from a single follicle will have a nett number of embryos corresponding to the number of corpora lutea and will therefore appear to have suffered neither loss nor polyovuly. Polyovuly, on the one hand, and pre-implantation loss on the other will be observable only in litters in which they do not compensate each other; that is, in litters in which only the one or the other occurs and in those in which the one exceeds the other. Assuming that polyovuly is not correlated with pre-implantation loss, and is distributed at random between the litters that do, and that do not, suffer this loss, the proportion of each that is masked by the other can be estimated. Data are available for 2179 litters with implanted embryos. Of these eighteen, or 0·83%, showed an excess of implantation sites over corpora lutea, 1365, or 62·64%, showed neither loss nor excess, and 796, or 36·53%, showed loss. A close estimate of the total percentage of litters in which polyovuly had occurred will be

$$\frac{100 \times 18}{1365} = 1{\cdot}32.$$

It follows that the percentage of litters in which the apparent loss has been reduced by polyovuly is $1{\cdot}32 - 0{\cdot}83 = 0{\cdot}49$. Thus the error introduced into the estimates of prenatal mortality from this cause is small and for most purposes may be neglected.

The eighteen litters showing polyovuly included sixteen showing an excess of one each, and two showing an excess of two each. Four other litters were observed in which the number of unimplanted embryos recovered by perfusion of the Fallopian tubes or uteri exceeded the number of corpora lutea in the ovaries by one each, but these were not included in the calculation, which was confined to litters with implanted embryos, because the experimental error introduced by failure to recover all the embryos by perfusion in stages less than 7 days' pregnant is too great. Since the mean size of litter in the sample of 2179 litters available was 5·76 and polyovuly occurs in 1·32% of the litters, it must result from 0·23% of follicles which ovulate.

Transmigration of ova. The passage of ova liberated from one ovary to the opposite uterus in wild rabbits was noted previously (Brambell, 1944). Since the two uterine horns have separate cervical canals transmigration of ova cannot take place by way of the cervix in rabbits. Therefore any that occurs must be by way of the peritoneal cavity. There were, in all, fifty-five litters in which the number of embryos on one side exceeded the number of corpora lutea in the corresponding ovary, though the total number of embryos on the two sides did not exceed the total number of corpora lutea in the pair of ovaries. Of these, eleven were in animals less than 7 days' pregnant, and forty-four were in animals at least 7 days' pregnant. The latter were derived from a sample of 2179 litters with implanted embryos, of which they represent 2·02%. In these there was evidence of the migration of two ova in each of two animals only, and of one ovum in each of the remaining forty-two animals, or 0·37% of ova ovulated.

The migration was from right to left in twenty-eight instances and from left to right in twenty-seven, so that the probabilities of migration occurring in either direction appear to be equal.

The only alternative explanation to transmigration that can be offered to explain these data would be the occurrence of polyovuly on one side accompanied by pre-implantation loss on the other. Since it has been calculated that polyovuly accompanied by pre-implantation loss occurs in only 0·49% of litters, and since the gain and loss would fall on opposite sides respectively in half of the cases, this explanation would account for less than one-eighth of the number observed.

The experimental error of counts of placental sites. Implantation of the blastocyst of the rabbit is of the central type and attachment to the uterine wall takes place early on the seventh day of gestation. Thereafter the blastocyst expands rapidly, and the swellings on the anti-mesometrial side of the uterus that mark the implantation sites can be clearly distinguished macroscopically by the middle of the seventh day. They are large and conspicuous by the middle of the eighth day and increase in size continuously thereafter, as development proceeds, until parturition. There is, therefore, no possibility of omitting to count swellings that contain living embryos after the seventh day. Moreover no precise information is available regarding the persistence during pregnancy of swellings in which the embryos have died. Such swellings

certainly begin to regress at once and, equally certainly, such regressing swellings can be distinguished amongst those containing developing embryos in a proportion of uteri at all stages after implantation, including those approaching full-term. The problem is whether a swelling in which the embryo has died soon after implantation can regress sufficiently to become indistinguishable before parturition. Experiments are being carried out to determine the rate of regression when all the embryos *in utero* are killed simultaneously at a given stage of development, and the results will be published elsewhere shortly. These experiments show that the swellings that contained embryos killed at $10\frac{1}{2}$ and $15\frac{1}{4}$ days are still visible at least at $17\frac{1}{2}$ and $24\frac{1}{4}$ days respectively. It is probable that the rate of regression is more rapid when the whole litter is destroyed, for then the animal comes into oestrus again soon, even before the swellings have disappeared, than it is when only some of the embryos are affected and pregnancy is maintained. It is hoped to investigate experimentally this problem at a later date. Meanwhile the possibility remains that if some embryos die

Table 5. *Distribution of number of placental sites in a litter according to stage of pregnancy*

No. of placental sites	Stage of pregnancy in days			
	11–15	16–20	21–25	26–32
11	1	—	—	—
10	1	—	—	2
9	5	8	4	4
8	28	19	14	14
7	62	71	42	46
6	128	117	90	100
5	136	112	84	123
4	92	66	71	136
3	58	35	29	43
2	11	7	4	16
1	4	2	1	7
Total	526	437	339	491
Mean	$5{\cdot}209 \pm 0{\cdot}067$	$5{\cdot}419 \pm 0{\cdot}070$	$5{\cdot}257 \pm 0{\cdot}076$	$4{\cdot}923 \pm 0{\cdot}067$
Difference	$0{\cdot}210 \pm 0{\cdot}097$		$0{\cdot}162 \pm 0{\cdot}103$	$0{\cdot}334 \pm 0{\cdot}101$

sufficiently soon after implantation their placental sites may disappear completely before the remaining embryos are born. If placental sites do disappear during gestation then the mean number of placental sites observed in the uteri at successive stages of pregnancy should decline, since no more can be formed. All the data of the distribution of the number of placental sites, both those containing living embryos and those regressing, in litters, grouped in four successive stages, are given in Table 5, together with the mean size of litter and its standard error in each group. The differences of the means of successive groups and the standard errors of the differences are given also. It will be seen that the differences between the 11–15- and the 16–20-day groups, and between the 21–25- and 26–32-day groups are significant, being more than twice and thrice their standard errors respectively. Thus there is a significant falling-off in the mean size of litter after the maximum in the 16–20-day group which can be accounted for by assuming the disappearance of some reabsorption sites. The significance of the initial rise in the mean size of litter from

the 11–15- to the 16–20-day group is very difficult to understand, and no satisfactory suggestion as to its meaning can be offered. Comparison of Table 5 with Table 2 shows that the successive changes in the mean numbers of corpora lutea and of placental sites during pregnancy closely parallel each other. This is brought out more clearly in Fig. 3. Assuming that this is due to a similar rate of disappearance, then the decline in the number of placental sites will tend to compensate the decline in the number of corpora lutea when both are used to estimate the prenatal loss. Such estimates, based on the difference between the numbers of corpora lutea and of placental sites, irrespective of whether the latter contain developing or regressing embryos, are estimates of the loss before implantation. Therefore estimates of the loss suffered before implantation in the surviving litters will tend to be unaffected by

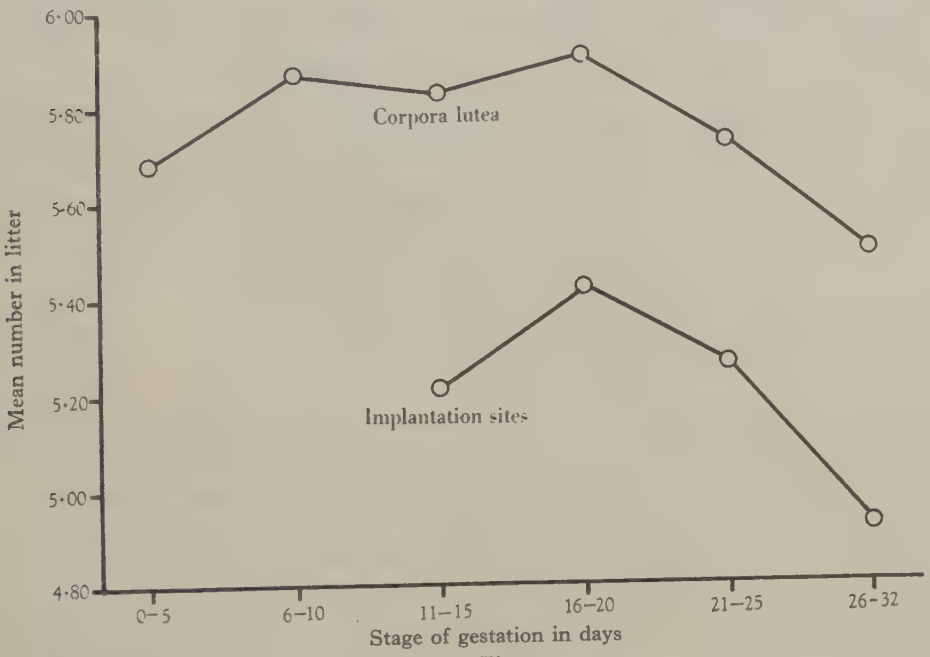

Fig. 3

the disappearance of corpora lutea and of placental sites. Estimates of the loss suffered after implantation are based on the difference between the number of developing embryos and of placental sites, and estimates of the total loss during pregnancy on the difference between the number of developing embryos and of corpora lutea. Since, by definition, developing embryos cannot disappear until parturition, both these estimates will be affected by the disappearance of placental sites and of corpora lutea respectively, being underestimates by the amount of this.

Independence of prenatal loss occurring before and after implantation

It has been indicated that the prenatal loss suffered before implantation can be distinguished from that suffered after implantation in any visibly pregnant animal by comparison of the number of placental sites with the number of corpora lutea and

of developing embryos respectively. The problem of whether the loss suffered after implantation is connected with that suffered before implantation, or is independent of it can be tested. It might be supposed that some or all of the factors which contribute to loss before implantation would continue to operate after implantation and to occasion further loss then; in other words, that an animal which has suffered loss before implantation would be predisposed to suffer further loss after implantation. If this were so, a greater proportion of those animals which had suffered loss, than of those which had suffered no loss before implantation, would suffer loss after implantation. If this were not so, the litters that suffered loss after implantation would be distributed between those that had suffered loss before implantation, and those that had not, in proportion to the relative numbers in these two groups. The available data from the various series are given in Table 6. The expected numbers

Table 6. *Coincidence of loss before and after implantation in each series*

Series	Total no. of pregnancies	No. showing no loss	No. showing loss before implantation only	No. showing loss after implantation only	No. showing loss before and after implantation	Expected
01	90	58	12	15	5	3·8
0	102	49	22	18	13	10·6
1	322	142	93	55	32	33·8
2	123	57	27	26	13	12·7
3	311	158	90	37	26	23·5
4	302	111	77	67	47	46·8
5, 6	346	195	83	45	23	20·8
7, 8	386	171	125	56	34	37·1
Total	1982	941	529	319	193	186·5

showing loss before and after implantation, calculated on the assumption that the loss after implantation is independent of that before implantation, is given in the right-hand column. The observed values diverge from the expected values for the whole sample by only 6·5. Testing this, $\chi^2 = 0{\cdot}48$, $n = 1$ and $P = 0{\cdot}5$. Therefore it must be assumed that the loss after implantation is independent of that before implantation. The expectation expressed as a percentage is represented graphically in Fig. 4 in the form of a square 10×10, and the deviation of the observed from the expected values is shown. Further, it can be seen from Table 6 that the observed do not diverge significantly from the expected values in any series, taken alone, the values of P ranging from 0·2 to 0·95.

The independence of the loss before and after implantation has been tested also for each age group from implantation to full-term. The results are shown in Table 7, and the observed do not differ significantly from the expected values in any group, $P = 0{\cdot}2 - 0{\cdot}8$. There are therefore no grounds for supposing that any fraction of the loss after implantation occurring at a particular stage of development is connected with the pre-implantation loss.

Finally, the distribution of those litters in which all the implanted embryos had died and were in process of regression in relation to loss before implantation was examined. The results given in Table 8 show that the loss of whole litters after implantation is also independent of the loss before implantation, $P = 0{\cdot}7 - 0{\cdot}8$.

Thus no relation has been discovered between the loss before and after implantation respectively, and it is legitimate, therefore, to treat them as independent and to examine them separately. This separation of the prenatal loss into two independent fractions had not been recognized when the earlier reports (Brambell, 1942, 1944) which dealt only with the total loss up to the time of examination, were published. The separation of these two fractions has thrown light on several problems that were previously obscure.

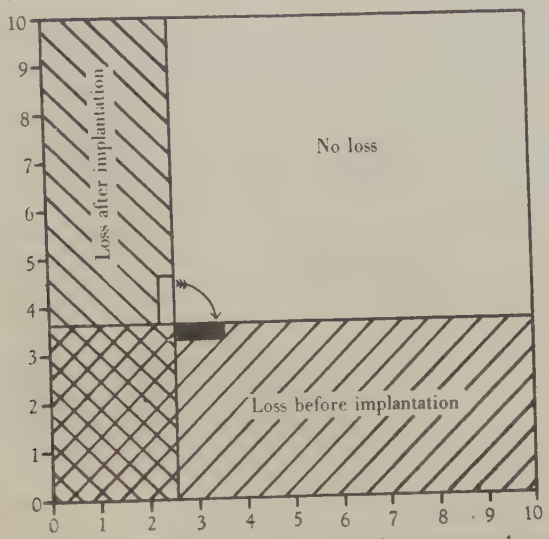

Fig. 4. Coincidence of loss before and after implantation expressed as a percentage. The black rectangle represents the divergence of the observed from the expected values.

Table 7. *Coincidence of loss before and after implantation at successive stages of pregnancy*

Stage (days)	Total no. of pregnancies	No. showing no loss	No. showing loss before implantation only	No. showing loss after implantation only	No. showing loss before and after implantation	Expected
7–10	320	188	99	18	15	11·8
11–15	473	114	68	181	110	109·5
16–20	394	198	102	57	37	33·2
21–25	306	179	96	18	13	11·0
26–32	448	261	162	18	7	9·4
Unaged	41	1	2	27	11	12·0
Total	1982	941	529	319	193	186·5

Table 8. *Coincidence of loss of whole litters after implantation with loss before implantation*

	Total no. of pregnancies	No. showing no loss	No. showing loss before implantation only	Total loss of litter after implantation only	No. showing loss before, and total loss of litter after, implantation	Expected
All data	1982	1162	663	98	59	57·2

SUMMARY

1. The technical problems underlying the estimation and analysis of the total prenatal mortality in a mammal are reviewed, with particular reference to the rabbit.

2. The material consisted of 7137 wild rabbits, of which 5089 were females. These comprise eleven series, according to the year, locality and treatment, particulars of which are given.

3. The experimental error in counts of corpora lutea was investigated and the technique critically examined. The changes in size of the corpus luteum during pregnancy were measured and a growth curve constructed. The mean number of corpora lutea in a litter, as determined from macroscopic counts, was found to be significantly lower at the beginning and end of pregnancy than in the middle. If this is due to omissions in the counts it represents a maximum error of 6·8% of corpora lutea. Microscopic counts of the corpora lutea in freehand sections of 180 pairs of ovaries of pregnant animals were made as a control. These revealed a total error in the original counts of 6·4% of the corpora lutea, affecting 25·6% of the litters, but this was distributed evenly throughout gestation.

4. Corpora lutea atretica formed from unruptured follicles are present in 16% of the pairs of ovaries, and comprise less than 2% of all corpora lutea. They are formed at the same time as the normal corpora lutea, and there is no evidence that any are formed subsequently during pregnancy in the rabbit.

5. It is estimated that 0·23% of the follicles which ovulate produce two embryos through the liberation of two ova.

6. Transperitoneal migration of 0·37% of ova, affecting 2% of the litters, was found to occur. The probability of migration either from left to right or from right to left appeared to be equal.

7. Significant changes in the mean number of implantation sites in the uteri counted at successive stages of pregnancy were observed, and particularly a decline at the end of gestation. They are of the same order as those in the mean number of corpora lutea, which they will tend to compensate so far as estimates of loss of ova before implantation are concerned. It is by no means clear that these are due to experimental error in the counts.

8. Estimates of the loss of embryos before implantation are based on the difference between the number of corpora lutea in the ovaries and of implantation sites in the uteri, and estimates of the loss after implantation on the difference between the number of implantation sites and of developing embryos. No significant relation has been found between the loss before and after implantation respectively, and it is concluded that the loss suffered after implantation is distributed independently of that which occurs before implantation.

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STUDIES ON STERILITY AND PRENATAL MORTALITY IN WILD RABBITS

II. THE OCCURRENCE OF FIBRIN IN THE YOLK-SAC CONTENTS OF EMBRYOS DURING AND IMMEDIATELY AFTER IMPLANTATION

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(With Plate 11)

1. INTRODUCTION

It has been shown (Brambell, 1942, 1944) that all the embryos in many of the litters of wild rabbits from Caernarvonshire die between the 11th and 15th days of gestation. The heaviest mortality is on the 11th and 12th days. A precisely similar mortality has been found to occur in wild rabbits from Anglesey, Norfolk and Dumfriesshire (unpublished), so that it must be regarded as widespread throughout Britain. The incidence of the mortality varies both from one locality to another and also from season to season in the same locality. The embryos that die at this stage of development are not aborted but undergo a gradual process of removal by autolysis *in situ*, and even after the embryos have disappeared the placental sites, where such 'reabsorption' has occurred, can be distinguished readily from post-partum placental sites or abortion sites by the remnants of the maternal decidual tissue projecting into the lumen of the uterus from the mesometrial side.

This particular kind of prenatal mortality is characterized both by the remarkable constancy of the stage of development at which it occurs and by the fact that it affects all the embryos in the litter almost simultaneously. The statistical significance of these characteristics has been discussed elsewhere (Brambell, 1942, 1944; Brambell & Mills, 1944, 1946). Attempts to investigate the immediate cause of the mortality embryologically have met with difficulty. The material was obtained largely by trapping and partly by shooting and netting (Allen, Brambell & Mills, 1946), and the pregnant animals had been dead for periods varying from an hour or two to a day or more before dissection. Further, the mortality could only be identified with certainty when the embryos had not only died but had begun to autolyse before the death of the mother. It has proved impossible in these circumstances to distinguish the autolytic changes consequent on the embryonic mortality and the putrefactive changes ensuing from the death of the mother from the irregularities in development preceding and leading to the death of the embryos. Since the incidence of the mortality is so high in some series it is reasonable to suppose that a substantial proportion of the embryos obtained on the 8th, 9th and 10th days of gestation would

have died on the 11th or 12th days if the mother had survived, and that therefore irregularities in development that would ultimately result in death might be detected in them without the complication of autolytic changes. An embryological investigation of such embryos and of their embryonic membranes was undertaken on this assumption. The investigations resulting, embryological and experimental, are the subject of this paper.

A brief outline of the salient points in the development of the rabbit from the 7th to the 15th day post-coitum, in so far as they are material, is necessary for clarity. The blastocysts reach the uterus from the Fallopian tube at the end of the 3rd day post-coitum when they are still very small and at about the time when the primitive yolk-sac cavity appears. They remain free in the uterine lumen for 3 days, during which time they are expanding with increasing rapidity. The blastocysts are very thin-walled vesicles, 3–4 mm. in diameter, still surrounded by the stretched and attenuated zona pellucida by the end of the 6th day. The fluid contents of the primitive yolk-sac cavity, the only cavity so far developed, accounts for the greater part of their bulk, and it is the expansion of this cavity which is mainly responsible for their rapid increase in size. At this time they fill and expand the uterine lumen, so that each is lodged in a small spherical chamber, the implantation cavity. Early on the 7th day the zona pellucida is shed and the naked trophoblast of the lower hemisphere of the yolk-sac adheres to the uterine mucosa on the antimesometrial side. The embryonic disk is orientated mesometrially. Thus implantation is of the central type and occurs early on the 7th day when the blastocyst has attained a diameter of 3–4 mm. It expands very rapidly during the two succeeding days and is about 12 mm. in diameter on the 9th day. This expansion is brought about partly by growth of the embryonic cells but mainly by increase of the yolk-sac fluid. It results in the antimesometrial wall of the implantation cavity being stretched thin and blown out like a bubble so that this part of the uterus becomes semi-transparent. Meanwhile the embryonic entoderm has been extending around the primitive yolk-sac cavity, forming with the overlying trophoblast the bilaminar omphalopleur, and converting the cavity into the definitive yolk-sac cavity. The establishment of the bilaminar omphalopleur is completed when the entoderm has extended to the ventral pole of the yolk-sac on the 8th day. Then the yolk-sac cavity is lined throughout with a very thin endothelium of entoderm cells, whereas previously a part of its wall consisted of trophoblast only. The bilaminar omphalopleur of the ventral or antimesometrial hemisphere of the yolk-sac wall is a transitory structure which disappears by the 14th day, thus placing the yolk-sac cavity in continuity with the uterine lumen and exposing the entoderm of the vascular yolk-sac splanchnopleur forming the dorsal hemisphere. This hemisphere of the yolk-sac wall meanwhile has been invaginated within the yolk-sac cavity, owing to the growth of the embryo and the extension of the exocoele, and it now forms an absorptive yolk-sac placenta.

The coelom appears by the beginning of the 8th day and is followed immediately by the formation of the amniotic folds. These close, completing the formation of the amnion, at $9\frac{1}{2}$ days post-coitum. The chorionic trophoblast of the outer walls of the amniotic folds adheres to the uterine epithelium, invading it and growing

into the mouths of the uterine glands, even before the amnion closes. This mesometrial attachment is the beginning of the allanto-chorionic placenta, which is rapidly established thereafter. Blood-islands differentiate in the area vasculosa at 8 days post-coitum, and blood first appears in the heart early on the 9th day. Presumably the heart has begun to beat at this time.

2. MATERIAL

The embryological material sectioned is shown in Table 1. Complete series of sections were not prepared with the majority of the embryos, but only a few sections through the middle of each uterine swelling were mounted. A few complete series were employed, mainly of the tame rabbit embryos. Most of the sections were stained with Ehrlich's haematoxylin and eosin.

Table 1

Age in days	Wild rabbits		Tame rabbits	
	No. of litters	No. of embryos	No. of litters	No. of embryos
7	29	29	—	—
7½	—	—	7	11
8	18	20	2	2
8½	3	3	4	4
9	5	6	8	8
9½	6	7	2	2
10	3	3	2	2
10½	5	5	2	3
11	—	—	1	1
11½	2	2	—	—
12½	1	1	—	—
Total	72	76	28	33

3. EMBRYOLOGICAL DESCRIPTION

During 1944, attention was directed particularly to the examination of embryos at stages of development between implantation and the time of maximum mortality. This was done with a view to distinguishing abnormalities in 7–10-day embryos that might result in their subsequent death. Consequently, all the swellings were opened under saline and the blastocysts examined in the fresh state with a binocular dissecting microscope. Previously it had been the practice to fix the swellings before opening them.

It was found that in a substantial proportion of pregnant uteri examined at these stages the contents of the yolk-sac cavities of the embryos were gelatinous rather than fluid. When the uteri were opened under saline and the blastocysts exposed, these often appeared translucent, rather than transparent, and when they were punctured they did not collapse, as do normal fresh blastocysts. It was the practice to pipette a few drops of absolute alcohol on to opened blastocysts of these ages, as this rendered the embryos more clearly visible and so assisted in ageing them accurately. However, this treatment, when applied to a gelatinous blastocyst, rendered the yolk-sac contents opaque and completely obscured the embryo which lay below it on the mesometrial side. Closer examination then revealed an irregular

network of white strands and trabeculae traversing the yolk-sac cavity. The earliest stage of development at which such gelatinous blastocysts were observed macroscopically was 7 days, and the latest was 12 days, but they were most frequent at 9 and 10 days. Sometimes all the blastocysts in a pair of uteri were similar in this respect, but in others a few only were affected.

Another abnormality was observed in many uteri consisting of semi-opaque opalescent strands, attached to the embryonic membranes and extending up and down the uterine lumen, but not adhering to the uterine wall. Sometimes a strand ended freely, but frequently it stretched from the membranes of one embryo to those of the next, connecting them together. Often the membranes of all the embryos in a uterus were so connected. These strands were sufficiently solid to remain intact while the uterus was pinned out and opened under saline, and they could then be stretched slightly and tested gently with a forceps. They varied in thickness up to a maximum of approximately 3 mm. in diameter. In a few instances they were tubular and filled with blood, though there was little or none free in the uterine lumen. The presence of the strands was associated commonly with deflation of the embryonic membranes. This deflation of the embryonic vesicles resulted in change in shape of the uterine swellings containing them, which, from being almost spherical and sharply delimited from the adjoining regions of the uterus, became elongated and tapered down to the diameter of the uterus between the implantation sites. Such swellings were often irregular in size and tended to appear dull and opaque, lacking the limpid translucence antimesometrially of swellings containing healthy embryos. It was often possible, with experience, to predict correctly from these indications that strands would, or would not, be found when the uterus was opened. The earliest stage at which strands were observed was 8 days and the latest was 12 days. They were most frequent at 8 and 9 days. They occurred at the same stages as the gelatinous blastocysts, and both abnormalities were frequently encountered together in the same uterus.

Some of the embryos of 8 or 9 days which displayed one or other or both of these abnormalities appeared to be otherwise normal and healthy, but in other instances no embryo could be distinguished macroscopically. The majority of uteri at 10, 11 or 12 days which contained strands or gelatinous material had retrogressing embryos. There are therefore grounds for suggesting that the gelatinous blastocysts and strands, though tending to occur at slightly earlier stages, are related to the mortality that attains a maximum on the 11th and 12th days.

Although many examples of both these abnormalities were observed in series 3–9 inclusive, complete data are available only for series 7 and 8. These are summarized in Table 2.

The shift of maximum numbers from the group of normal litters on the 7th day to the group showing one or both abnormalities on the 8th and 9th days, to those showing both regression and abnormalities on the 10th day, and finally to those showing regression only on the 11th and 12th days is significant, and clearly suggests that the abnormalities are a phase in the process of regression. It is equally possible, however, to interpret these data as showing that the gelatinous condition of the

yolk-sac contents and the formation of strands are either a change that precedes the death of the embryos at 10, 11 or 12 days, or that they are characteristic of embryos which have already died and are regressing.

Microscopic examination of sections of blastocysts revealed that the gelatinous condition of the yolk-sac contents was due to the presence of a branching network of fine fibrils (Pl. 11) which stained faintly with eosin after Bouin's fluid fixation. Sometimes they formed a very open-meshed reticulum throughout the yolk-sac. Sometimes a number of spherical foci could be discerned scattered unevenly in the yolk-sac cavity, each consisting of a denser central mass of fibres with irregularly branching fibres radiating from it (Pl. 11, fig. 2). Sometimes what appeared to be an autolysing cell or the pycnotic remains of a nucleus could be discerned in the centre of such foci, in others it was represented by a small cavity, and in many no central structure could be discerned. Sometimes the reticulum was compacted into a dense felting of fibres in which the individual ones could scarcely be distinguished (Pl. 11, fig. 1). Such felted masses were usually confined to the periphery of the yolk-sac cavity,

Table 2

Age days	No. of litters				
	Showing neither abnormalities nor regression	Showing abnormalities only	Showing both regression and abnormalities	Showing regression only	Total
7	16	1	0	0	17
8	4	9	1	0	14
9	5	13	3	0	21
10	6	3	5	4	18
11	5	0	2	6	13
12	6	0	1	15	22
Total	42	26	12	25	105

frequently in the antimesometrial hemisphere, and the rest of the cavity was occupied by a much more open reticulum. Sometimes strands or trabeculae of tangled or felted fibres stretched across the cavity, and it was plainly these which were visible macroscopically.

The individual fibres varied in diameter, the thickest being of the order of 0·5 μ. They branched irregularly and the free extremities tapered gradually. No visible structure could be discerned in them. They were visible after treatment with several different fixatives, but Bouin's fluid gave the best results. They were difficult to stain, the most satisfactory results being obtained with eosin, and with water blue in Passini's stain. They were found in smears of yolk-sac content, as well as in sections, both air-dried and fixed by several methods, and sometimes reacted positively, at other times negatively, to vigorous Gram staining.

The distribution of the reticulum was remarkable. It was present in the majority of embryos of 8 and 9 days examined microscopically, and in some of those of 7 and 10 days. It was not found in any blastocysts of 7 days or earlier which were still surrounded by the zona pellucida. The few 8- and 9-day embryos in which it was not found were badly preserved, and so it was not possible to be sure that it was

absent. It varied very greatly in the degree of development and in many was slight and localized, consisting only of a few fibres near the periphery of the yolk-sac cavity. Clearly these would not have been classed as gelatinous blastocysts on macroscopic examination. Within the embryo it was confined to the yolk-sac cavity and the gut cavity, in which it could be seen in a few instances. Since the mid-gut opens freely into the yolk-sac cavity at these stages that is not surprising. It was never observed in the exocoele or amniotic cavity, although these are only separated from the yolk-sac cavity by thin membranes. It was sometimes present also in the uterine lumen. It was found also, at slightly later stages, in 10- and 11-day embryos in the cavities or crypts of the placental region formed from the enlarged uterine glands after their necks had been invaded and blocked by the trophoblast.

Sections of strands joining blastocysts showed that these consisted of felted cords of similar fibres, crumpled remains of embryonic membranes, cell debris, leucocytes and red blood corpuscles of maternal origin, compacted together by the pressure of the uterine walls. Their structure was consistent with the view that they represent a later stage in the evolution of gelatinous blastocysts.

The fibrous network never appeared to have penetrated into the tissues, either embryonic or maternal, being always confined to the cavities named.

Examination of blastocysts of tame rabbits at corresponding stages of development did not reveal any that could be identified macroscopically as gelatinous blastocysts, nor were any strands connecting the blastocysts found. Microscopic examination of sections showed that the yolk-sac cavities of many of them were completely free from the fibrous network. It was present in others but was much less developed than in the majority of the wild embryos. Only in a few instances did the network attain the density and extent met with in unmistakable gelatinous blastocysts of wild animals. The fibres, when present, were so similar in structure and staining affinities to those in wild material that the identity of the two need not be questioned.

The suggestion that the fibrous reticulum was an artefact produced by the histological technique was inconsistent with facts, since the fibres were present both in sections and smears, and after a wide variety of fixatives had been employed. It was obvious that they were not tissue fibres formed by fibroblastic cells, since no such cells are present in the yolk-sac cavity. Two possibilities remained: first, that the reticulum was the mycelium of a micro-organism living in the yolk-sac cavity and the uterine lumen; secondly, that it was a network of organic fibres formed by some biochemical process without the intervention of fibroblastic cells.

The morphological resemblance to the mycelium of an actinomycete when grown in culture media was so close that the possibility of the reticulum being an invasive organism belonging to this group could not be eliminated microscopically. Since the ultimate objective was to elucidate the course of the prenatal mortality, the possibility of the presence of a causative organism could not be dismissed lightly and no other structure that could be interpreted as such had been observed in the uteri. Consequently it was decided to undertake culture experiments to determine if a micro-organism was involved.

4. BACTERIOLOGICAL EXPERIMENTS

Cultures were made from the yolk-sac contents of embryos from eighteen wild rabbits 6–13 days post-coitum. Several of the embryos used were typical gelatinous blastocysts, and the presence of the reticulum in the others was confirmed either by smear preparations from the same embryo or by sections of other embryos from the same uteri. Both plate and stab cultures were prepared, using a variety of media, and were incubated aerobically and anaerobically. Sterile conditions could not be obtained, since post-mortem material was employed, the animals having been gutted by the trappers and having been dead several hours before they were received. Therefore a variety of the usual contaminants appeared in the cultures, but no one type of micro-organism was present consistently. An actinomycete appeared in two cultures from one rabbit soon after the experiments were begun. It belonged to the group of *Micromonospora* and was microaerophilic when first obtained, but it rapidly became aerobic on subculturing. This positive result of the culture experiments, taken in conjunction with the close morphological resemblance of the reticulum in sections and smears, encouraged further attempts to isolate a causative organism, but it proved impossible to repeat the result.

Since the reticulum was known to be present in the yolk-sac content of some tame rabbit embryos, it was decided to attempt to obtain cultures from them under aseptic conditions. Cultures were made from the yolk-sac content of several embryos from each of eight tame rabbits 8 or 9 days pregnant. Aseptic precautions were taken and upwards of a hundred stab cultures made on blood agar and semi-solid glucose nutrient agar which normally prove suitable for actinomycetes. These were incubated both aerobically and anaerobically. The aseptic precautions proved adequate, and accidental contaminants only developed in less than 1% of the cultures, a satisfactorily small proportion. All the other cultures were sterile. No actinomycete was obtained in any of the cultures, although the presence of the reticulum was demonstrated in some of the smears of each animal used. The possibility of an actinomycete being present and unculturable with the technique employed was negligible. It was concluded therefore that the actinomycete originally obtained from the wild material was an accidental contaminant, and the reticulum observed in the yolk-sac was not a micro-organism culturable by the usual techniques. These results not only disposed of the theory that an actinomycete was present in the yolk-sac fluid but they provided very strong evidence that this fluid is bacteriologically sterile.

5. HAEMATOLOGICAL EXPERIMENTS

It appeared probable that the network in the yolk-sac cavity was composed of protein fibres, possibly fibrin, since it was neither an artefact nor a micro-organism. Consequently clots of fibrin were prepared from citrated rabbit plasma by the addition of calcium. These were fixed in Bouin's fluid, embedded in paraffin, sectioned and stained with eosin, using the same technique as was employed for the embryos. The fibrin network prepared in this way closely resembled the

reticulum in the yolk-sac both in microscopic appearance and in histochemical properties.

Experiments were planned to test whether fibrinogen was present normally in the yolk-sac fluid of embryos of tame rabbits. Since thrombin is specific in its action in converting fibrinogen into fibrin it was decided to employ it. Rabbit embryos of approximately 9 days post-coitum were used, as the content of the yolk-sac is then at a maximum. The embryos were obtained either from rabbits killed for the purpose or by unilateral hysterectomy under ether anaesthesia in the course of experiments designed for another purpose. The yolk-sac fluid was withdrawn separately from each embryo by means of a hypodermic syringe, the needle being inserted through the uterine wall antimesometrially. The contents was withdrawn into 0·1 ml. of 3% sodium citrate solution in a graduated 1 ml. syringe, and the citrated yolk-sac fluid measured and transferred to a Wasserman tube. Quantities of up to 0·75 ml. of yolk-sac fluid were obtained from each embryo. Care was taken

Table 3

Serial no. of rabbit	Age of embryos post-coitum	How obtained	No. of embryos tested	Clotting reaction	Subsequent history of remaining embryos
E 93	9 days 3 hr.	By laparotomy	1 1	+ +	One still living at 10 days 23 hr.
E 94	9 days 5 hr.	By laparotomy	1 1 1	+ + +	All regressing at 11 days 23 hr.
E 95	8 days 22 hr.	By laparotomy	1 2	+ +	Still living at 11 days 20 hr.
E 96	9 days 1 hr.	By laparotomy	1 3	+ +	Still living at 11 days 21 hr.
E 99	8 days 22 hr.	By laparotomy	1	+	Still living at 12 days 1 hr.
E 102	9 days 0 hr.	By autopsy	6	+	—
E 103	9 days 1 hr.	By autopsy	7	+	—

to avoid contamination of the fluid with blood, and any samples in which this occurred were rejected. It was impracticable to ensure by this means that the fluid contents of the yolk-sac so withdrawn was not contaminated with traces of other embryonic fluids from either the exocoele or the amnion, if this happened to be already formed, or with tissue exudation from the uterine lumen. It is certain that the amount of such contamination, if any, was small because the amount of fluid obtained was commensurate with the size of the yolk-sac cavity and because the deflation of the uterine swelling was observed as the fluid was withdrawn. A solution of human thrombin in saline, containing 2 units per 1 ml., was added, either to the undiluted citrated yolk-sac fluid, or to the fluid after it had been diluted with an equal quantity of saline. The quantity of thrombin solution added was such that the final concentration was 1 unit per 1 ml. This resulted in the formation of a clot in from 30 to 60 sec. at room temperature in all cases. The experiments are summarized in Table 3. The samples of yolk-sac fluid from two or more embryos from the same rabbit were combined in some instances.

Serial dilutions with saline were made containing $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, ..., $\frac{1}{64}$ of yolk-sac fluid and were clotted by the addition of equal quantities of the thrombin solution. These were compared visually with clots formed in similar serial dilutions of rabbit-blood plasma. It was possible to estimate by this means that the concentration of fibrinogen in the yolk-sac fluid was of the order of 50% of that in the blood plasma. The quantities of yolk-sac fluid that could be obtained from the embryos of one litter would be ample, on this basis, for the estimation of nitrogen in the clot by micro-analyses. Accordingly clots were prepared from the combined yolk-sac fluid of several embryos of the one litter. The citrated fluid was diluted with equal quantities of saline, so as to reduce the density of the clot, and thrombin solution added. After the clot had formed it was frozen in a refrigerator and subsequently thawed at room temperature. This caused the clot to contract to a comparatively small size. The residue was filtered off on a fine sinter-glass filter and the clot washed several times with saline and then with distilled water. The wet clot was dried in a vacuum. The filtrate and washings were combined and the nitrogen estimated both in the residue

Table 4

Reference no. of mother	Age of embryos post-coitum	No. of embryos employed	Analysis per 1 ml. of yolk-sac fluid			
			Weight of dried clot mg.	Amount of N in dried clot mg.	% of N in dried clot	Amount of N in residue of yolk-sac fluid mg.
E 95	8 days 22 hr.	2	1·96	0·22	11·4	5·17
E 96	9 days 1 hr.	3				4·90
E 102	9 days 0 hr.	6	1·87	0·20	10·9	6·37
E 103	9 days 1 hr.	7	2·86	0·31	10·7	5·64
Mean	—	—	2·16	0·24	11·1	5·52
Plasma	—	—	5·82	0·73	12·6	9·64

and in the dried clot. The results are given in Table 4, the estimate of nitrogen in the residue being corrected for the small amount added in the thrombin solution. Estimates of nitrogen in a single sample of rabbit-blood plasma treated in the same way are given for comparison. The sample of plasma was taken from a rabbit in late pregnancy (29 days). Another sample, not included in the table, was taken from a rabbit in early pregnancy ($9\frac{1}{2}$ days) and yielded 5·78 mg. of dried clot per ml. of plasma, an amount which agrees closely with that given in the table. Weight for weight of dried clot the mean concentration of fibrin in the yolk-sac fluid is 37% of that in the sample of plasma, and the corresponding figure based on the nitrogen content of the clot is 33%. The difference in the concentration of fibrin in the yolk-sac fluids of the embryos of E 102 and E 103 respectively is well beyond the experimental error of the determinations and is clearly significant. The differences in the percentages of nitrogen in the dried clots obtained from yolk-sac fluid may be accounted for by a reasonable margin of error in the determinations, but the difference between these and the percentage of nitrogen in the plasma clot appears significant, but may be due to inefficiency in the technique of separation of the clot from the residue. It can scarcely be due to inequality in the drying of the clots, since all

three of the determinations on yolk-sac fluid agree reasonably well, while that on plasma is in excess of them. It would be necessary to assume that none of the clots from yolk-sac fluid had been completely dried, and that approximately the same amount of moisture had been left in each to account for the difference on this basis. The mean quantity of nitrogen in the residue of the yolk-sac fluid, after the removal of the clot, is 57% of that in the serum. Further inquiry is necessary before any suggestion can be offered as to the nature of the nitrogen-containing compounds in the residue of the yolk-sac fluid. It may be noted, however, that the proportion of fibrin-nitrogen to non-fibrin-nitrogen in the yolk-sac fluid and in the plasma is different.

6. DISCUSSION

The results reported in this paper demonstrate that fibrinogen is a normal component of the yolk-sac fluid of rabbit embryos and that at 9 days post-coitum it is there in quantities of 30–40% of that in the blood plasma. Since the appearance of fibrinogen in the yolk-sac cavity precedes and accompanies the establishment of the embryonic vascular system it is highly probable that it is of maternal origin. It would be very difficult to believe that it could be produced in such quantities by the embryo at so early a stage of development. If this assumption is justified then the fibrinogen must be absorbed through the yolk-sac wall. The trophoblast of the yolk-sac wall at least must be readily permeable to it. At this time the trophoblast of the antimesometrial hemisphere of the yolk-sac adheres to and is actively invading and destroying the uterine epithelium, presumably passing the products of the destruction of the maternal tissues into the yolk-sac cavity. The results indicate that among the proteins maternal fibrinogen at least can traverse the trophoblast as such. It is not clear whether the fibrinogen can also pass through the entoderm of the yolk-sac wall for it has been found in the cavity soon after the establishment of the bilaminar omphalopleur has been completed. It is possible, therefore, that all the fibrinogen enters the yolk-sac cavity before this has been completely enclosed by entoderm.

The nutriment derived by the mammalian embryo in the uterus from the mother is divided conventionally into histiotrophe and haemotrophe. The histiotrophe consists of the secretions of the tubal and uterine glands, transudation, extravasated blood, cell debris and other products of destruction of the maternal endometrium by the trophoblast. The haemotrophe consists of nutritive materials carried in the maternal blood and transferred from the maternal to the foetal circulations in the placenta. Therefore until the foetal circulation is established and the placenta is functional the nutrition of the embryo is entirely histiotrophic. After the placenta has begun to function it is predominantly haemotrophic. Histiotrophic nutrition may continue after the establishment of haemotrophic nutrition but is of progressively declining importance.

The stage of development at which haemotrophic nutrition begins varies in different groups of mammals according to the type of implantation and placentation. It tends to be late in species with central implantation, in which the blastocyst

remains free in the uterine lumen for a comparatively long time and reaches a relatively large size before it becomes attached to the uterine wall. The rabbit is one of these, and in it haemotrophic nutrition cannot begin until late on the 9th or early on the 10th day after copulation. The efficiency of the placenta as an organ of haemotrophic nutrition will depend on the barrier presented by the intervening tissues to the transfer of nutritive materials from the maternal to the foetal circulations, which are never in direct contact. Primitively six tissues are involved in this barrier; the maternal capillary endothelium, connective tissue and uterine epithelium and the foetal trophoblast, mesenchyme and capillary endothelium. The classification of placentae suggested by Grosser (1927) and commonly employed is based on the extent of the reduction in these tissue layers. The greatest possible efficiency is attained in the haemo-endothelial type of placenta in which only the foetal endothelium intervenes between the maternal and foetal blood. This type of placentation was recognized first by Mossman (1926) in the rabbit. It results from the destruction of all the intervening maternal tissues by the placental trophoblast, so that the maternal blood circulates through lacunae in the spongy trophoblast, bathing its surface directly, and in the subsequent thinning out and disappearance of the trophoblast and mesenchyme covering the foetal capillaries at the points where they adjoin these lacunae. According to Mossman (1926) this condition is achieved in the rabbit on the 13th or 14th day. Thus the maximum efficiency of the haemotrophic method of nutrition is attained at the time when the bilaminar omphalopleur disappears and the entoderm of the vascular splanchnic wall of the yolk-sac is exposed to the uterine lumen.

The problem of placental permeability has been the subject of many researches, which are admirably summarized and discussed by Needham (1931) and need not be recapitulated. Extraordinarily little is known, however, regarding the passage of substances into the embryo at earlier stages, when the nutrition is exclusively histiotrophic. Evidently fibrinogen can enter at this stage. Much larger quantities of nitrogen also are present in the yolk-sac fluid in some other form than fibrinogen, but the nature and derivation of these nitrogen-containing compounds awaits elucidation. Yet if fibrinogen can enter as such it is quite probable that other maternal proteins can do so as well. Presumably the fibrinogen is utilized by the embryo, possibly being digested in the yolk-sac cavity and absorbed in soluble form by the entoderm.

The increase in size of the blastocyst from the 7th to the 9th day is very rapid and is due mainly to increase in volume of the yolk-sac fluid. The blastocyst is not more than 4 mm. in diameter at the time of attachment on the 7th day and attains a size of approximately 12 mm. in diameter on the 9th day, when as much as 0·75 ml. of fluid can be aspirated from the yolk-sac. The increase in volume during the two intervening days therefore is of the order of twenty times. Throughout this period of rapid expansion the blastocyst remains almost spherical, being only slightly elongated in the longitudinal axis of the uterus and somewhat flattened on the mesometrial side, presumably these being respectively the directions of least and greatest resistance to expansion by the uterine tissues. How a sufficient hydrostatic

pressure is generated to expand the uterine chamber and stretch thin its muscular wall is difficult to imagine. Yet this occurs within the vesicular blastocyst bounded only by a very thin cellular membrane that is highly permeable to protein.

It is not practicable to aspirate fluid from the cavities of the amnion and exocoele, and to test it with thrombin for the presence of fibrinogen, at such early stages of development of the embryo when these cavities are newly formed and still very small. Microscopic examination of sections of embryos of wild rabbits in which the yolk-sac contents were clotted has shown that fibrin is always absent from the amniotic cavity and the exocoele. It must be concluded that their walls are impermeable to fibrinogen. This difference in permeability to protein of the omphalopleur and the other embryonic membranes is interesting. It may be suggested that the mesoderm which encloses both the exocoele and the amniotic cavity, but does not enter into the bilaminar omphalopleur, provides the barrier.

The conversion of the fibrinogen into a clot of fibrin in the yolk-sac cavity must be regarded as an abnormality, since it had not occurred in many obviously healthy tame rabbit embryos examined. The problem of when the clot forms in the yolk-sac cavities of embryos of wild rabbits is difficult to solve. Some of the wild rabbits in which gelatinous blastocysts were found were examined very soon after death, while the bodies were still quite warm. Clots only form in the yolk-sac cavities of healthy tame rabbit embryos when these are left in the gutted carcass of the mother for many hours, and even then they tend to be much less apparent. These facts indicate that clotting of the yolk-sac fluid often occurs in wild rabbits before the death of the mother. Moreover, the variability often experienced in the degree of development of the clot from embryo to embryo in the same uterus of a wild rabbit bears out this conclusion. Whether or not clotting occurs before the death of the embryos, when this precedes the death of the mother, is still more obscure. Yet this problem is important in relation to the prenatal mortality, for if, as might seem the simplest assumption, it only occurs after the death of the embryos, then all the gelatinous blastocysts observed in the wild material were already dead and would represent a heavy prenatal mortality, occurring at 8, 9 and 10 days, not previously recognized, in addition to that which undoubtedly occurs about 11 and 12 days. If, on the other hand, the clotting precedes the death of the embryos, then the gelatinous blastocysts observed can be regarded as abnormalities in development preliminary to the subsequent death and regression of the embryos on the 11th and 12th days, and it is not necessary to assume a mortality additional to that already recognized. It would appear that this problem can be solved only by experimental means, and investigations are in progress along such lines. Preliminary results indicate that the embryo can survive clotting of the yolk-sac contents at least for a short time. It follows that should clotting prove to be preliminary to the death of the embryos around the 11th and 12th days, then knowledge of the factors which bring it about might throw much light on the cause of this important fraction of the prenatal mortality in wild rabbits.

Finally, it will be apparent that knowledge of the nutrition of the mammalian embryo during the histiotrophic phase is extraordinarily scanty. Little is known

regarding the composition of the histiotrophe or the physiological factors governing its production, the manner of its passage through the limiting membrane of the embryo, and the way in which it is subsequently utilized. It is hoped that this paper will serve to draw attention to these problems and to show that the yolk-sac fluid of the rabbit embryo provides convenient material for physiological and biochemical investigations of them. There is here a wide field for fundamental research of great potential significance in relation to prenatal health.

7. SUMMARY

1. The yolk-sac contents were observed to be gelatinous in many embryos of wild rabbits at from 7- to 12-day stages, but particularly at 8 and 9 days. Gelatinous strands were observed in the uterine lumen, often connecting adjoining blastocysts, in 8–12-day stages, but particularly at 8 and 9 days. Gelatinous blastocysts and strands frequently were encountered together in the same uterus.

2. Evidence is adduced that these abnormalities, though tending to occur at slightly earlier stages, are related to the mortality which attains a maximum on the 11th and 12th days.

3. Microscopic examination revealed the presence of a reticulum of fine fibrils in the yolk-sac cavity, the histological characters of which are described. The degree of development of this reticulum varied widely from one uterus to another, and often in the individual embryos of the one litter. The presence of this reticulum, when sufficiently dense, was responsible for the gelatinous character of the yolk-sac contents. The gelatinous strands consisted of felted masses of these fibres and of maternal and foetal tissue debris.

4. The fibrous reticulum was absent from the cavities of the amnion and exocoele.

5. A similar reticulum was present sometimes, though rarely so well developed, in the embryos of tame rabbits. Frequently it was absent.

6. It was shown by histological means that the reticulum was not an artefact. The alternatives, that it was either a micro-organism or a network of organic fibres, remained.

7. Morphologically the reticulum resembled the mycelium of an actinomycete so closely that the possibility that it was an invasive organism belonging to this group could not be ignored. Culture experiments disproved this theory and provided satisfactory evidence that the yolk-sac cavities of tame rabbit embryos containing the reticulum were bacteriologically sterile.

8. Sections of clots of fibrin prepared from rabbit-blood plasma presented a similar histological appearance.

9. The presence of fibrinogen in the yolk-sac fluid of 9-day embryos of tame rabbits was demonstrated by clotting the aspirated and citrated fluid with thrombin. Comparison of the clots formed in parallel series of dilutions of the yolk-sac fluid and of plasma indicated that the concentration of fibrinogen in the former was of the order of 50% of that in the latter.

10. Separation of the fibrin from the residue of the yolk-sac fluid was effected by filtration and repeated washing. The mean weight of dried fibrin obtained per ml.

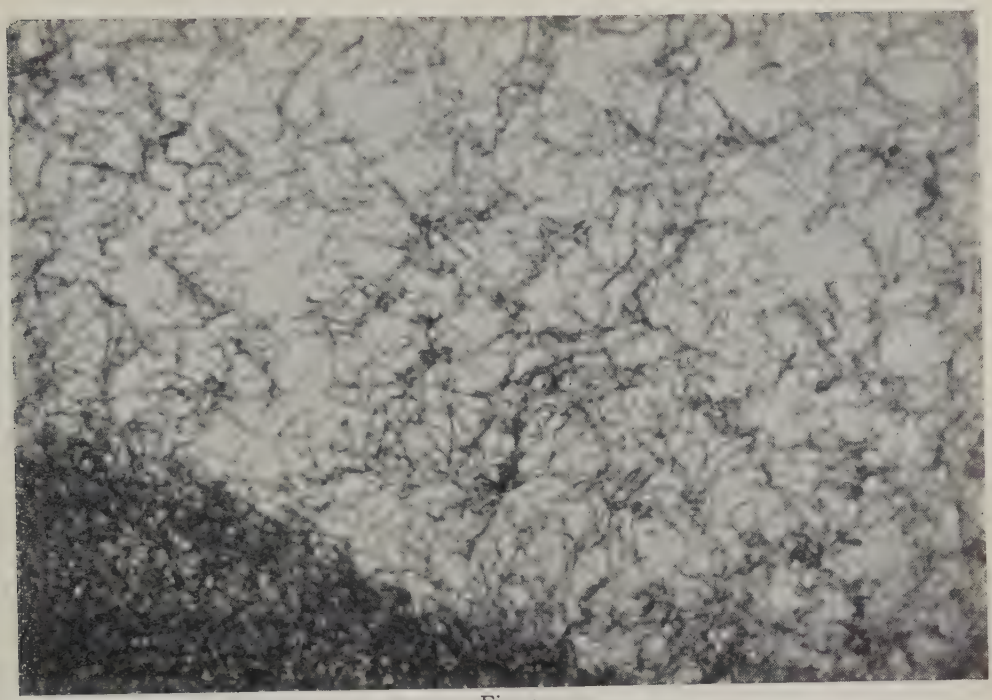

Fig. 1

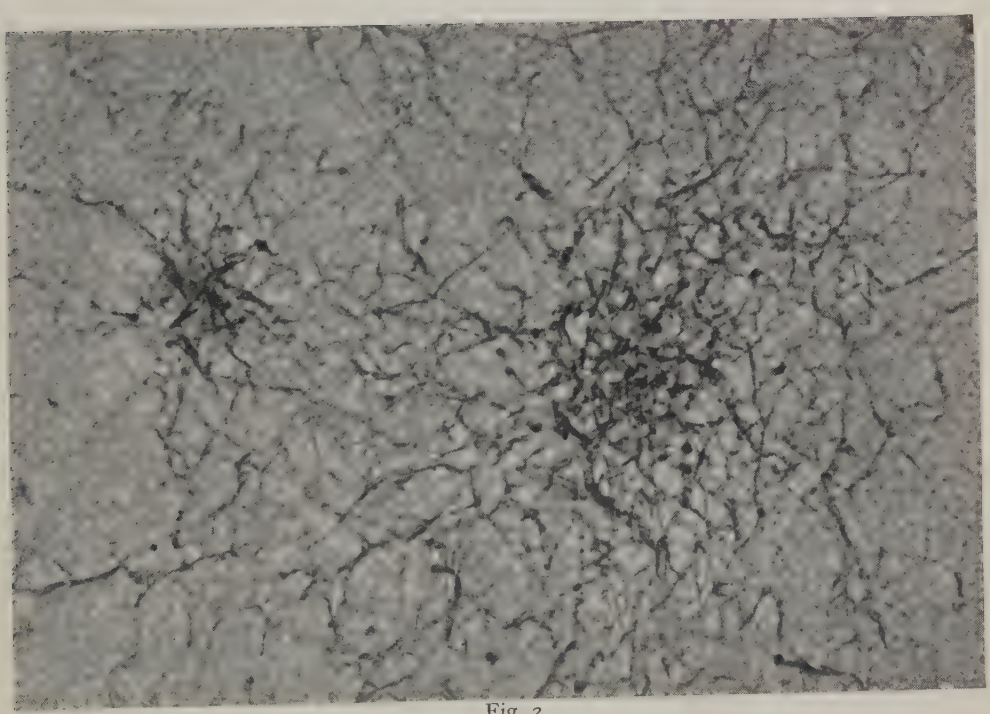

Fig. 2

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of yolk-sac fluid was 37% of that from plasma and its nitrogen content was 33%. The amount of fibrin in the yolk-sac fluid varied significantly from litter to litter. The mean quantity of nitrogen in the residue of the yolk-sac fluid was 57% of that in the blood serum.

11. The significance of these results in relation to embryonic nutrition and prenatal mortality is discussed.

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EXPLANATION OF PLATE 11

Fig. 1. Fibrin reticulum in the yolk-sac cavity of an embryo (6/271, 1 R) of a wild rabbit, *circa* early 8 days post-coitum. The reticulum is felted into a dense mass at the bottom of the figure. × 680.

Fig. 2. Fibrin reticulum in the yolk-sac cavity of an embryo (6/129, isolated blastocyst) of a wild rabbit, *circa* 8 days post-coitum. Two foci are seen with fibrillae radiating irregularly from them. × 680.

THE PRODUCTION OF FATTY ACIDS IN THE ALIMENTARY TRACT OF THE DOG

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The production of a mixture of acetic, propionic, and butyric acids is a characteristic feature of alimentary fermentation in the ruminant and in the horse, pig, rabbit, and rat. The proportions of these acids present in the digesta of the large intestine, and of the stomach in the case of the ruminant, is similar in these species, with the exception of the rat, in which acids higher than acetic form a bigger proportion of the whole. In order to complete the series, the investigation was extended to include the dog, to see (*a*) whether alimentary fermentation was appreciable in a carnivorous animal, and (*b*) whether the products of fermentation were the same.

METHODS

Experimental

Four dogs were used. They were fed on a diet consisting of one-third meat and two-thirds bread for at least a week before the experiment. Dogs 1 and 2 were fed within 2 hr. of the experiment, dog 3 received its last meal the morning before the experiment, while dog 4 received its last meal the evening before the experiment.

Dog 1 (18·7 kg.) was aged, dog 2 (16·4 kg.) and dog 3 (10·2 kg.) were probably 1–4 years old, and dog 4 (8·0 kg.) was approximately 6–8 months old.

The animals were anaesthetized with Nembutal by intravenous injection. The abdomen was opened and blood was withdrawn from vessels draining the stomach, small intestine, and the large intestine and in some cases the portal vein. Blood was also taken from the carotid artery and the jugular vein. After death, the whole alimentary tract was removed and divided into stomach, anterior two-thirds of small intestine, posterior third of small intestine, and large intestine. The contents of each portion were weighed and sampled in the manner previously described (Elsden, Hitchcock, Marshall & Phillipson, 1946).

Analytical

The procedures adopted for the determination of volatile acids in the digesta were identical with those previously described. In all cases, however, distillates were redistilled according to the method of Friedemann in the presence of acid mercuric sulphate in order to remove any formic or lactic acids present.

Volatile acid in the blood was distilled by the method described by McClendon (1944) with the exception that three 30 ml. fractions were collected and titrated. This was found to be necessary after the distillation of known quantities of acetic

acid. Proteins of blood were precipitated by the addition of 3 c.c. of a freshly prepared solution of 25% metaphosphoric acid to 5 c.c. of blood already luked in water in a 50 ml. volumetric flask. The volume was made up to 50 ml. and 5 or 10 c.c. of the filtrate were used for distillation. This method was found by Elsden (personal communication) to be more reliable than that recommended by McClendon.

RESULTS

The values for volatile acids in blood show clearly that more is present in the blood leaving the large intestine than in any of the other venous and arterial samples collected. This is clear proof that absorption of volatile acid occurs from the large gut. In dog 2, the evidence is incomplete as technical errors spoiled the samples of peripheral blood that were taken. This dog defaecated immediately before anaesthetization and its colon was practically empty during the experiment. This is reflected by the fact that the figure found for blood draining the colon is no greater than that found for the stomach.

Table 1. *ml. 0·01-N volatile acids per 100 ml. blood*

Dog ...	1	2	3	4
Carotid	1·4	—	2·1	6·9
Jugular	0·9	—	1·7	5·5
Stomach	2·8	6·3	—	—
Small intestine	2·7	2·8	2·4	12·0
Large intestine	9·8	6·3	11·7	43·0
Portal	—	—	2·5	6·4

Analysis of the digesta in the various parts of the alimentary canal support this finding, for appreciable quantities of volatile acid are present only in the digesta of the large gut, as shown by Table 2.

Table 2. *Total volatile acid as g. acetic acid*

	After 1st distillation				After 2nd Friedemann distillation			
Dog	1	2	3	4	1	2	3	4
Stomach	0·212	0·227	Empty	0·072	0·135	0·104	Empty	0·064
Small intestine First two-thirds	0·021	0·025	0·077	0·017	0·013	0·015	0·034	0·009
Small intestine Last one-third	0·022			0·031	0·010			0·017
Large intestine	1·403	0·057	0·507	0·249	1·383	0·048	0·482	0·200
Faeces	—	0·795	—	—	—	0·780	—	—

The values obtained after the second Friedemann distillation, that is the figures for volatile acid after the removal of formic and lactic acids, are expressed in Table 3 as a percentage of the dry matter of the digesta. The concentrations found in the large intestine are appreciable and are comparable to those found in the large intestine of herbivores.

Table 3. *Volatile acid as g. acetic acid per 100 g. dry matter*

Dog	1	2	3	4
Stomach	0·13	0·07	—	0·15
Small intestine First two-thirds	0·25	0·23	0·14	0·155
Small intestine Last one-third	0·41			0·58
Large intestine	4·92	0·23	5·26	3·33
Faeces	—	2·71	—	—

The figures are surprising in that the values for volatile acid found in the blood draining the large gut are highest in dog 4, although the total amount of volatile acid present in the digesta of this organ is lower in this animal, and the concentration less than that of dog 1 and dog 3.

The bulk of the volatile acidity found in the digesta of the large intestine is apparently due to fatty acid other than formic acid, as the volatile acid obtained after the second Friedemann distillation was in no case less than 80% of the total volatile acidity of the first distillation. This was not so with the remainder of the digesta, as the reduction here was in most cases in the region of 50%.

Chromatographic analysis, according to the method of Elsden (1946), of the acids obtained from the digesta of the large intestine gave the following results:

Table 4. *Partition of volatile fatty acids of the large intestine*

Dog ...	1	2	3	4
Butyric acid %	15·8	15·7	16·9	2·4
Propionic acid %	37·8	28·0	34·3	42·3
Acetic acid %	46·8	56·3	49·8	55·3

The identity of the individual acids was not confirmed by analysis other than the speed at which they passed through the column of silica gel, but their behaviour in this respect was the same as that of the acids named in Table 4.

In order to make the results comparable to those previously obtained in other animals, the total volatile acidity after the first distillation is given in Table 5 as g./kg. The figures show that the production of volatile acids is insignificant compared to production in herbivorous animals and is less than that in omnivorous

Table 5. *Volatile acid as g. acetic acid per kg. body weight*

Dog 1	0·09
Dog 2	0·02
Dog 2 + faeces	0·11
Dog 3	0·05
Dog 4	0·21

animals such as the pig and the rat on this basis; but when it is recalled that the concentration of volatile acid in the colon varied between 3·3 and 5·3 g. acetic acid per 100 g. dry matter (Table 3), figures that fall in the middle of the range found

in the caeca of herbivorous animals, it appears probable that the principle circumstance which limits the part played by fermentation in the digestion of the dog is the size of the large intestine: otherwise there is nothing to distinguish the dog from the ruminant in the intensity of fermentation when this is judged by the concentration of volatile acid found in the digesta.

The concentration of volatile acid found in the blood draining the large gut was equal to that found in blood draining the rumen in one case and in the remainder was higher than that in peripheral blood. Absorption therefore does occur and the acids are not all excreted in the faeces.

SUMMARY

The significance of fermentation in the large gut of the dog has been investigated. According to the quantities of volatile acids and their relation to body weight, the lower fatty acids do not appear to contribute more than a small portion of the energy requirements of the animal. Evidence is presented to show that the individual acids produced in the large intestine consist largely of acetic and propionic acids and that the amount of butyric present is small. This is the same mixture of acids found where fermentation occurs in the alimentary tract of ruminants, horses, pigs, rabbits and rats. The higher proportion of propionic acid is interesting and suggests that the propionic acid bacteria are normal inhabitants of the large intestine of the dog and possibly of a wide variety of animals.

Thanks are due to Miss J. F. New for her assistance in some of the analytical procedures used in this work.

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DISK-SPHERE TRANSFORMATIONS PRODUCED BY LYSINS, AND THEIR REVERSAL, IN SYSTEMS OF DIFFERENT *p*H

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It is now recognized that lysis is preceded, in the case of nearly all haemolytic systems containing mammalian red cells, by a disk-sphere transformation which is at first reversible but later irreversible. While this shape change is an event which occurs during the course of reactions leading up to haemolysis, its occurrence seems to be described by relations not altogether the same as those which are applicable to the description of the haemolytic phenomena themselves. Thus it has already been shown that in the case of some lysins the shape change does not occur until just before the cell haemolyses, whereas in the case of other lysins it takes place almost as soon as the cells are added, although lysis does not occur for minutes or even hours. One concludes from this that the reactions which produce the shape changes are not identical with those which bring about the lysis, but have a certain degree of independence of the latter. The purpose of this paper is to describe two other differences which distinguish the shape transformation from the phenomenon of lysis which usually follows it. The first of these is that a change in *p*H does not usually affect the shape change and the subsequent lysis in the same way, for in the case of most lysins haemolysis is inhibited by an increase in *p*H in the range 6·0 to 8·5, whereas the shape transformation occurs more readily at high *p*H's than at low ones. The second is that substances which inhibit the haemolytic process may or may not transform the spherical form into the disk, an observation which suggests that the shape transformation is not simply an early manifestation of the effects of the same processes which eventually bring about haemolysis.

I. THE SHAPE IN ISOTONIC CITRATE-PHOSPHATE BUFFERS AT VARIOUS *p*H's

The cells of 8 ml. of freshly obtained heparinized human blood are washed three times with 1% NaCl, and are then suspended in isotonic citrate-phosphate buffers of various *p*H so that each ml. of suspension contains 10^8 red cells. Citrate-phosphate buffers are selected because red cell shape is so well maintained in 3% sodium citrate, and because the requisite *p*H range can be obtained by varying the proportions of *M*/15 KH_2PO_4 and *M*/15 Na_2HPO_4. In preparing the suspension media, the buffers are prepared first and then mixed with equal volumes of 3% sodium citrate: it is the *p*H of these mixtures which are given in the Tables.

The cell suspensions, in citrate-phosphate buffers at *p*H 6·0, 6·6, 7·3 and 8·3, are added to 1% NaCl in the proportion of 0·5 ml. of suspension to 2 ml. of

1% NaCl. The shape of the cells is ascertained by examining them immediately in uncovered drops on plastic slides: this examination is repeated at intervals, the cell-buffer system being kept at 25° C. throughout.

Discoidal red cells in dilute suspension can be distinguished macroscopically from spherical cells if the contents of the tube containing the system are first mixed, and if the tube is then given a sudden flip in order to set up currents in the fluid in it. For a second or two, a watered-silk appearance is observed when the cells are discoidal, this being caused by the flat cells being carried along in the currents and reflecting light in different directions as they move. Crenated disks give the same effect in a less striking way; spheres and crenated spheres give no such effect at all. By repeatedly comparing the shape, as judged by this macroscopic method, with the shape as seen microscopically, considerable skill can be gained in determining shape from the appearance of the system in the tube, and the approximate lysin concentrations in which disks become spheres can be found in this way. For more exact observations in the region of these concentrations, the cells should always be examined under the microscope so as to verify the results obtained macroscopically.

The results of experiments designed to ascertain the shape of human red cells in isotonic citrate-phosphate buffers at various *p*H's are given in Table 1, which shows that there is a tendency for the discoidal form to crenate and ultimately to become spherical at the higher *p*H's.* Information of this type is essential for the interpretation of the results of the experiments in §§ II and III.

Table 1. *Shape of washed human red cells in citrate buffers after various times of standing at* 25° C.†

Observed	*p*H 6·0	*p*H 6·6	*p*H 7·3	*p*H 8·4
At once	Disks	Disks	Disks	Disks with slight crenation
After 30 min.	Disks	Disks	Disks	Crenated disks and spheres
After 1 hr.	Disks	Disks	Disks	Crenated spheres
After 5 hr.	Disks	Disks	Crenated disks	Spheres

† The *p*H given is that of the citrate-phosphate buffer, and not that of the completed system. The *p*H of the cell suspensions are 6·0, 6·5, 7·0 and 7·9 respectively.

II. SPHERING AND LYSIS PRODUCED BY VARIOUS LYSINS AT VARIOUS *p*H's

Four identical series of dilutions of the lysin (e.g. saponin) in powers of 2, are set up in a series of tubes, each finally containing 2 ml. of the diluted lysin. The range over which the dilutions have to be made so as to include both the dilution in which complete lysis occurs and that in which perfect sphering occurs has to be found by trial, but can be inferred from the values in Table 2 for the five substances

* These results differ from those described by Teitel-Bernard (1932) because the systems and the conditions of observation are not the same as in his experiments. His systems contained plasma diluted about 1 in 40, which is sufficient to prevent sphering at *p*H 8·4. Further, the zone of crenation described between *p*H 7·0 and 6·0, corresponding to the intracellular crystallization of haemoglobin with its accompanying birefringence, was the result of observations made after some hours and not, as in the case of the experiments described here, after 30 min. only.

distearyl lecithin, sodium tetradecyl sulphate, sodium taurocholate, saponin and digitonin. These last three substances are both dissolved in and diluted with 1% NaCl. The distearyl lecithin is prepared as a sol in 1% NaCl; the tetradecyl sulphate is first dissolved in methyl alcohol in the proportion of 5 mg./ml., and is added to and diluted with 1% NaCl immediately before use.

Table 2. *Minimum quantities in γ required for sphering and for complete lysis of* 5×10^7 *washed human red cells in citrate-phosphate buffers at the end of* 30 *min. at* 25° C.

Lysin or sphering agent	*p*H 6·0		*p*H 6·6		*p*H 7·3		*p*H 8·4	
	Lysis	Spheres	Lysis	Spheres	Lysis	Spheres	Lysis	Spheres
Lecithin	—	15	—	5	—	3	—	0·5
C-14 sulphate	7	2	8	1	9	0·5	10	0·1
Sodium taurocholate	200	25	200	12	300	4	350	2
Saponin	12	10	12	10	12	10	16	2
Digitonin	5	4	5	4	6	5	6	1

To each tube in the first series of dilutions of each lysin is added 0·5 ml. of the red cell suspension in citrate-buffer at *p*H 6·0, prepared as in § 1, above. Similarly, 0·5 ml. of the cell suspensions at *p*H 6·6, 7·3 and 8·3, prepared as in § I, is added to each tube in the second, third and fourth series respectively of the dilutions of each lysin. The contents of the systems are mixed and allowed to stand for 30 min. at 25° C. The smallest quantity of each substance required at each *p*H to produce (*a*) complete lysis and (*b*) perfect sphering is then determined by examining the systems first macroscopically and then microscopically.

The results of a typical experiment of this kind are shown in Table 2.

III. REVERSAL EFFECTS

A series of systems are prepared containing 5×10^7 human red cells per ml. in citrate-phosphate buffer at *p*H 7·3 together with distearyl lecithin, sodium tetradecyl sulphate, sodium taurocholate, saponin, or digitonin in sufficient quantity to produce perfect sphering in 30 min. at 25° C. (column 2 of Table 2). The systems should be at least 20 ml. in volume. At the end of 30 min., 1 ml. portions of a system are transferred to small tubes, and various 'reversing substances' are added to reconvert the spherical cells into disks.

The reversing substances used in these experiments were two components of plasma (which itself converts spheres into disks if added before the stage of the prolytic sphere is reached), serum albumin and cholesterol, as well as one of the agents which has already been described (Ponder, 1942) as producing a reversal of the lecithin shape transformation, benzene. It may be stated at once that the other principal inhibitory components of plasma, lecithin and serum globulin, do not produce reversal of the shape change brought about by any of the five lysins used in these experiments.

The serum albumin (kindly sent to me by Dr E. J. Cohn as a 25% solution) is made up as 0·1 and 0·01% solutions in 1% NaCl. The cholesterol is prepared as

a sol in water with a cholesterol content of 500 γ/ml. (Lee & Tsai, 1942), and is diluted 1 in 10 with 1% NaCl just before use. The benzene is used as a saturated solution in saline (1 ml. = 780 γ).

The procedure is to take 1 ml. of a system containing a sphering agent or lysin, to examine the cells so as to be sure that they are spherical, and then to add a small amount of a reversing agent, e.g. 0·1 ml. of 0·01% serum albumin, and to ascertain if it converts the cells immediately into disks. If it does not, further small amounts of the reversing agent are added until the volume of the system is increased to about 1·5 ml.; if the cells are still spherical, another 1 ml. sample of the system is taken and 0·1 ml. of the reversing agent in higher concentration, e.g. 0·1% serum albumin, is added. If this is not enough to turn the disks into spheres, larger amounts and if necessary higher concentrations of reversing agent are added and the effect on shape, if any, observed. Care should be taken to control the observations by adding the same amount of 1% NaCl, in order to be sure that a mere dilution of the system does not turn the spheres into disks.

This procedure is repeated with the systems containing each of the five sphering agents, always in just sufficient concentrations to produce sphering in 30 min. at 25° C., and with each of the three reversing agents, the cells being suspended in the first instance in citrate-buffer at *p*H 7·3. The whole experiment is then repeated with cell suspensions at *p*H 6·0 and 6·6, the quantities of sphering agent being changed so as always to be the minimum amount for sphering in 30 min. at 25° C.; these amounts require to be determined beforehand by constructing a table such as Table 2. There is no point in repeating the experiments at *p*H 8·3, for at this *p*H the cells are almost spherical in the citrate-buffer itself, and the quantities of the sphering agents required to produce sphering are very small.

I have not been able to demonstrate any dependence on *p*H of the reversing effects, and so Table 3 shows the quantities of the three reversing agents required at *p*H 7·3 only.

Table 3. *Quantities of reversing agents, in γ, required for the reversal of sphering of 5×10^7 cells after 30 min. at 25° C. and at p*H 7·3

Sphering agent	Amount per 5×10^7 cells	Serum albumin	Cholesterol	Benzene
Lecithin	3	150	No reversal	550
C-14 sulphate	0·5	15	75	780
Sodium taurocholate	4	50	No reversal	780
Saponin	10	25	No reversal	No reversal?
Digitonin	5	100	No reversal	No reversal?

In the case of each of the five lysins, a reversal of the disk-sphere transformation is brought about by serum albumin in quantities of from 15 to 150 γ. Only in the case of the lysin sodium tetradecyl sulphate does cholesterol produce reversal; in systems containing the other lysins it is ineffective at all *p*H's between 6·0 and 8·3. The uncertainty attached to the effects of benzene in systems containing saponin and digitonin is due to the concentration range which separates the production of

spheres from the lysis of the cells being so small as to make it very difficult to pick up reversal effects with the amounts of benzene which can be introduced into the systems; these are limited by the solubility of benzene in saline.

IV. DISCUSSION

Three principal conclusions, all of interest in connexion with the phenomena of the shape transformations, can be drawn from the data in Tables 1, 2 and 3.

(1) *The mole ratios.* By converting quantities into moles, two additional tables can be constructed from the data. The first of these (Table 4) shows the number of

Table 4. *A.² of red cell surface, per molecule of lysin or sphering agent. Values calculated from the values in Table 2*

Lysin or sphering agent	*p*H 6·0		*p*H 6·6		*p*H 7·3		*p*H 8·4	
	Lysis	Spheres	Lysis	Spheres	Lysis	Spheres	Lysis	Spheres
Lecithin	—	67	—	200	—	350	—	2000
C-14 sulphate	60	210	53	420	89	800	42	4200
Sodium taurocholate	4	32	4	67	3	180	2	360
Saponin	110	130	110	130	110	130	80	670
Digitonin	330	420	330	420	270	330	270	1670

Table 5. *Ratios of reversing agent to sphering agent, calculated from Table 5*

Sphering agent	Serum albumin	Cholesterol	Benzene
Lecithin	0·57	—	110
C-14 sulphate	0·13	110	3600
Sodium taurocholate	0·10	—	800
Saponin	0·35	—	600
Digitonin	0·36	—	1500

A.² of red cell surface, per molecule of lysin or sphering agent, in systems in which (*a*) perfect sphering, and (*b*) complete lysis, occurs. The values in A.² are minimum values, for it is assumed that all the lysin or sphering agent in the system is distributed over the red cell surface; this is almost certainly not the case, considerable quantities being left free in the suspension medium (Ponder, 1946).

A point which stands out clearly is that some substances can produce the disk-sphere transformation, and can even bring about lysis, when they are present in such small quantities that they cannot cover the cell surface with a monolayer. There seems to be no foundation for the idea that a lysin must form a monolayer at the surface before it can bring about lysis (Gorter, 1937, cf. Ponder, 1941). The anionic detergent sodium tetradecyl sulphate, for example, produces sphering when there is only one molecule per 420 A.² at *p*H 6·4 and per 800 A.² at *p*H 7·3, even if all of it is imagined to be monodisperse and present at the red cell surfaces. Since the dimensions of the molecule are about $20 \times 4{\cdot}5 \times 4{\cdot}5$ A., there is not enough of it to form a monolayer. The same remarks apply to the sphering produced by lecithin at *p*H 7·3.

Table 5 shows the value of the ratio

$$\frac{\text{Number of molecules of reversing agent needed to produce reversal}}{\text{Smallest number of molecules of sphering agent needed for sphering}}$$

for the five sphering agents and the three reversing agents. In the case of serum albumin (M.W. taken as 70,000) the ratio varies from 0·10 to 0·57, i.e. it is of the same order of magnitude for all the sphering agents. In the case of benzene, on the other hand, the ratio varies from 110 to 3600, which means that benzene is a very much poorer reversing agent than is serum albumin and that its effect of the spheres produced by the various sphering agents is not at all the same, even allowing for the fact that the determinations are made to the nearest power of 2 only. In the single case in which cholesterol produces a reversal of the disk-sphere transformation, the ratio has the value of 110; like benzene, cholesterol is therefore a much poorer reversing agent than serum albumin, as well as being one whose effect is less general. Serum albumin, indeed, seems to occupy a unique position which may be related to its being the 'anti-sphering substance' for the disk-sphere transformations which occur, as a result of a *p*H change, between glass slides and coverglasses (Furchgott, 1940 *a*, *b*; Furchgott & Ponder, 1940).

(2) *p*H *dependence of shape change and of lysis.* The data in Table 2 show that the *p*H dependence of the sphering produced by four of the five substances examined (distearyl lecithin does not produce lysis except in very high concentrations and then only partially) is not at all the same as the *p*H dependence of the haemolysis which follows it. The amount of lysin required to complete the haemolytic process increases with increase in *p*H, but the amount required to bring about the disk-sphere transformation decreases with increase in *p*H. The shape changes in the systems containing lysins are no doubt contributed to by the tendency towards spontaneous crenation and sphering at the higher *p*H's (Table 1), but the conclusion remains that the processes which bring about haemolysis are affected by *p*H changes in a different way from those processes which result in the shape change, even although the latter are usually associated with the early stages of the former.

(3) *Reversal of shape transformation and inhibition of lysis.* The observation that a reversal of the disk-sphere transformation produced by lecithin occurs when a large amount of saline is added to the system (Ponder, 1936) suggests that the reversing agents may act by reacting with the lysins and reducing their concentration. This simple explanation is not tenable in view of the fact that cholesterol, a powerful inhibitor of lysis by the anionic detergents, sodium taurocholate, saponin, and digitonin, does not bring about a shape reversal except in the case of the spheres produced by the C-14 detergent. The effect of the reversing agents on shape must therefore be regarded as something specific, and as distinct from their effect on the concentration of free lysin in the system.

SUMMARY

1. Although sphering almost always occurs at some time before a mammalian red cell haemolyses, the shape change has to be placed in a variable position on the time scale to which the progress of the lytic reaction is referred. In the case of some lysins, e.g. saponin, the shape change is a late event, while in the case of others, e.g. sodium tetradecyl sulphate, it is an early event on the same time scale.

2. In the case of four lysins (sodium taurocholate, sodium tetradecyl sulphate, saponin and digitonin) the amount of lysin required for complete haemolysis increases with increasing *p*H, but the amount required to produce sphering decreases with decreasing *p*H.

3. Serum albumin produces a reversal of the disk-sphere transformation brought about by distearyl lecithin, sodium taurocholate, sodium tetradecyl sulphate, saponin and digitonin. Benzene has a weak reversing effect when the spheres are formed as the result of the action of the first three substances mentioned, and cholesterol produces reversal of the shape change only when it is brought about by sodium tetradecyl sulphate. The simple explanation that the reversing agents act by reacting with the lysins and reducing their concentration is not tenable, and the effect of the reversing agents must be regarded as something distinct from their inhibitory effect on the lysin in the system.

4. Some of the substances used can produce the disk-sphere transformation and can even bring about lysis when they are present in such small amounts that they cannot cover the red cell surface. Either the effects produced by each molecule of sphering agent or lysin extend to neighbouring regions on the cell surface and perhaps also into the interior, or sphering and lysis occur as the result of changes at spots on the surface.

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STUDIES IN THE DEVELOPMENT OF THE RAINBOW TROUT (*SALMO IRIDEUS*)

I. THE HEAT PRODUCTION AND NITROGENOUS EXCRETION

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(With Five Text-figures)

I. INTRODUCTION

Animal development can be studied as a transfer of energy from non-living unorganized reserves to a living organized embryo. The egg is a small amount of protoplasm with a supply of supplementary material—the yolk—which varies considerably in amount in different kinds of eggs and from which there arises an embryo containing food reserves insignificant in amount. It is possible to draw up a balance sheet of the energy reserves in the system and the energy lost by the system between the beginning and the end or between any two arbitrary points in time throughout development. This is attempted in the present study of the development of the trout embryo from hatching until the end of the yolk-sac period when, under normal conditions, the alevin begins to eat.

Earlier studies of the energy metabolism of the embryo are reviewed in Needham (1931, § 7). Heat production during a substantial proportion of the period of development was measured for the chick by Bohr & Hasselbalch (1900, 1902) and in the toad by Gayda (1921). Bohr & Hasselbalch's work is alone comparable with the present study in that the heat production observed was related to the metabolism. In the chick the main energy source is fat and deductions about its metabolism could be drawn from measurement of oxygen uptake and carbon dioxide output. Needham attempted an extended correlation between chemical and energy metabolism largely based upon this work, that of Murray (1925) and his own observations. Murray had concluded from his fuel values of the chick embryo that, in the early stages at least, the usual coefficients for relating chemical analysis and fuel value did not obtain. Needham, accepting this conclusion, was able to outline the framework of development for both chemistry and energetics, but to do this observations of several workers had to be extrapolated and adjusted. It has been the aim of the present study to accomplish sufficient experimental work on the same or similar batches of material by the same person in the hope of securing a more unified and consistent picture of the relationship between the chemistry and the energetics of development.

It will be shown that during the whole cycle of development in the trout the usual constants which relate (1) nitrogen content to protein content, (2) protein to fuel value and (3) fat content to fuel value can be applied within the limits of error of the several techniques used in the investigation.

II. MATERIAL

Observations were begun in the breeding season of 1933 and continued for the four succeeding years, the last sequence of measurements relating to the period December 1936 to March 1937. In all, nine batches of eggs were investigated, and with each repetition, the range of observation was extended. Much reduplication has resulted from this method of repetition with amplification, and, for publication, only those data relating to the last batch of eggs, upon which the most complete series of observations were made, will be used. Certain observations made on earlier batches have been used for interpreting results obtained later from other materials—a procedure which may be criticized. Conditions of rearing were as far as possible identical for all batches. The complete tables of observations of work up to March 1936 are given in the Thesis for the Ph.D. degree deposited in Cambridge University Library.

The series of measurements made upon the developing egg-alevin system were:

I. *Upon the living material:*

(*a*) the rate of heat production—sometimes daily, invariably every second day—from the first day after fertilization to the end of the yolk-sac period;

(*b*) the rate of ammonia and urea excretion over roughly the same period as the heat production measurements.

II. *Upon fresh material.* Determination of:

(*c*) wet weight of embryo and yolk;

(*d*) dry weight of embryo and yolk;

(*e*) total carbohydrate content of the whole system.

III. *Upon dried samples from* II (*d*). Determination of:

(*f*) the fuel values of embryo and yolk by combustion in a bomb calorimeter;

(*g*) the total nitrogen content of embryo and yolk samples;

(*h*) the total material extractable by carbon tetrachloride.

The determinations I (*a*) and (*b*) and II (*c*), (*d*) and (*e*) were made over the same period on the same batch of eggs. The results of analyses in § III are used to interpret the 1936–7 observations. Since a large quantity of material is necessary for the combustion in § III (*f*) a large batch was reared specially for the purpose.

The trout egg was selected for this study for several reasons:

(1) eggs can be obtained in relatively large numbers from one female;

(2) eggs can be fertilized almost simultaneously;

(3) eggs are easily reared and survive sorting into weight groups;

(4) greater uniformity in development may be secured by selecting those which hatch within a limited period;

(5) moribund eggs can be detected with ease;

(6) the embryo does not feed for about eighty days when reared at 10° C.;

(7) the embryo can be separated by dissection from the yolk-sac and without contamination with yolk. (Since embryos are small, large numbers are required for a single determination; this minimizes the effect of sampling errors.)

The rainbow trout (*Salmo irideus*) eggs were reared in hatchery trays kept in a constant temperature room at $10 \pm 0{\cdot}5°$ C., supplied with a continuous flow of water maintained at a temperature of $10 \pm 0{\cdot}2°$ C. for the 80-odd days of the experiment. The water supply came from a tank at constant level and its temperature was recorded continuously. The eggs were sent 'green'; that is immediately after fertilization, from the Surrey Trout Farm Hatchery at Nailsworth, Glos. Mr Steven of the hatchery selected the eggs from one female and reserved samples of the same eggs unfertilized. From the fifth to the twelfth day any shock disturbs gastrulation overgrowth: only part of the yolk is surrounded. This results in the death of the embryo. Once overgrowth is complete, however, deaths are infrequent until hatching, at which time some die. After hatching, alevins are negatively phototactic and must be reared in complete darkness otherwise they kill themselves in an endeavour to swim through the hatchery trays.

III. METHODS

(*a*) *The determination of wet and dry weight*

As they form the connecting link between all analyses on dried samples and observations on the living egg and alevin, the determinations of wet and dry weights are fundamental to subsequent treatment. Unless the contrary is indicated all observations at whatever stage in development apply to the system embryo + yolk-sac = alevin, and not to the egg. At as early a stage as possible before hatching the alevin was removed by dissection from the egg membrane or 'chorion'. This operation is facilitated if the eggs are hardened in 4% formalin before dissection; but comparisons showed that the water content of the embryo may be changed to a significant degree by such fixation. Accordingly all figures quoted refer to the fresh unfixed material.

Wet weights of groups of five alevins were determined in the following way. The material was dried superficially on a Buchner funnel in a current of air after rinsing rapidly with 96% alcohol, and transferred to an open watch-glass. At a fixed period of time from the rinsing, the weight was taken. They were then washed with water on to a clean porcelain tile, and the yolks separated by dissection using cataract knives in the water drop. Under these conditions the yolk rounds off cleanly. No attempt was made to weigh the yolks directly; their weight was obtained by difference. The dissected embryos after being dried superficially and weighed as before, were then dried in a porcelain dish to constant weight in an electric oven at 100·5° C. The yolks were left to dry on the tile, removed as fine flakes by scraping, transferred

to small porcelain dishes, dried as before, cooled in a desiccator and weighed as rapidly as possible. Both dried embryo and yolk material are very hygroscopic.

(*b*) *Heat production*

The technique of heat production measurement was a modification of that of Hill (1911). Each microcalorimeter consisted of two selected vacuum flasks of approximately 200 ml. capacity with identical characteristic for heat conduction for a water content of 100 ml. One flask (the control) contained water alone while the other contained the experimental material with water sufficient to make a total volume equal to that of the contents of the control flask. The temperature difference between these flasks is measured by a thermocouple over some period of time, during which period production of heat in the experimental flask leads to a steady drift in the temperature difference between the flasks. The couples were calibrated against Beckmann thermometers to the nearest $\frac{1}{1000}$° C. Work on these lines was done for the three years 1934–5, 1935–6, 1936–7, with apparatus which was repeatedly modified; the description which follows refers to the final form (1936–7). The nature of the material necessitated that (i) measurements be conducted at 10° C., some 5–6° C. below normal room temperature, and (ii) a continuous supply of air for aeration be maintained. The flasks were fitted with glass collars so that the entire flask could be held below the surface of the bath. A stream of saturated air at the temperature of the bath was passed down glass tubes fitted with sintered glass filters to generate a stream of fine bubbles into each flask. Around these tubes three sets of soldered copper-constantin thermojunctions were constructed: two two-junction couples to record the temperature difference between each flask and the bath, and a four-junction couple to record the temperature difference between the flasks. Each tube thus carried twelve metal wires, and to reduce heat exchanges by conduction those copper wires which dipped into water were 42 s.w.g. copper wire. All couples were insulated with four coats of Parson's Bakelite yacht varnish (no. 27) kindly supplied gratis by the firm, each coat being baked to hardness at temperatures rising from 60 to 120° C. over a period of 9 hr. The couples and intake tubes were held steady in the flasks by loose wads of cotton-wool in the glass collars. The rate of aeration was adjusted to 1–2 bubbles a second as observed by bubble counters. Experiments were always performed in duplicate. The e.m.f. of the couples was measured by a short-period moving coil galvanometer of low resistance with an auxiliary all-copper resistance box in series. All switches and coils were of copper. The galvanometer was calibrated against a Weston standard cell and a potential divider giving 10^{-5} of the input. To avoid errors from shifting of the galvanometer zero, and to gain greater sensitivity reversal throws were measured. The optical system was arranged to work at 4 m. instead of at 1 m., the standard working distance. Care was required in handling the apparatus, as stray heating effects in the circuit may cause temporary and transient fluctuations larger than the actual measurements recorded. Experiments were rarely run for more than 10 hr. as the rate of heat production tends to fall off after this. In order to estimate the extent of heat losses, and so to correct the heat-production curves, the conductivity

of the experimental flask was determined both for heat losses and heat gains under working conditions at intervals throughout the course of the work. The conductivity factors were consistent to within 12–14% for each apparatus, and the means of all determinations were used in correcting the heat-production curves. The application of corrections for flask conduction followed Hill very closely. In no case did the total correction exceed more than 25% of the observed values. The error in a single experiment was well within 10%.

The characteristic conduction (K) is determined by a number of factors:

(1) conduction through the flask wall (identical in both flasks at 100 ml. content);

(2) exchange of heat between the air above the bath and the flask contents by conduction along the leads (if the couples are symmetrical the differential effect is minimal);

(3) the heating effect of the air current. This is difficult to control. The rate of bubbling is maintained as constant as possible, whilst heat losses from evaporation are reduced by aerating with saturated air. Since a pressure excess is required to produce air bubbles 3 cm. below the surface of the water in the flasks a small cooling effect is produced by aeration.

Each determination was made as follows. Fifty alevins were made up to a total volume of 100 ml. in a measuring cylinder and transferred to the experimental flask. Using the couple itself as recorder, ice-water was added from a graduated pipette until the temperature was a little below that of the bath and the control flask (a few thousandths ° C.), the corresponding volume of water being removed to keep the heat capacity and volume of the system constant. Couples were adjusted, wads of cotton-wool fitted, the rate of bubbling adjusted, the lid put over the thermostat bath, and the whole left for 30 min. to settle down. Readings were taken at convenient intervals for 8–9 hr. The temperature-time curve obtained was corrected for heat losses. Knowing the heat capacity of the system, the sensitivity of the galvanometer and the characteristics of the couple the absolute heat production may be calculated.

(*c*) *Fuel values*

The fuel values for dry embryo and dry yolk were determined by combustion in the standard Berthelot-Mahler bomb calorimeter. In this case the size of sample required for the analysis was large (more than 0·5 g.) and so a special batch of eggs was reared for these determinations. The dried yolk was of necessity burnt as a powder because on pressing it into a pellet fat is squeezed out. The apparatus was calibrated using benzoic acid. Experimental errors lay within 0·2%.

(*d*) *Nitrogen excretion. Method*

The method used for estimating ammonium ion and urea in dilute solution was developed from that given by Teorell (1932) for the estimation of ammonia in distilled water. Advantage was taken of the absorption of ammonia by permutit (Folin & Bell, 1917) in order to remove ammonia from solutions of ammonia and urea.

The method uses the reaction between ammonia and urea and alkaline hypobromite within the range of *p*H 8·5–9·0:

$$3NaBrO + 2NH_3 = 3NaBr + 3H_2O + N_2,$$

$$CO(NH_2)_2 + 8NaOH + 3Br_2 = 6NaBr + Na_2CO_3 + 6H_2O + N_2.$$

Each reaction is complete to within 0·5% for fixed concentrations of NaOH and Br_2.

Sodium hypobromite is added in excess of that required for the reaction and after a fixed period of time HBr is added and the bromine thus liberated is titrated with a *N*/2500 solution of naphthyl red (benzene-azo-α-naphthylamine). A second determination with NaBrO is done on a sample from which NH_3 has been removed by permutit.

If the blank is *B* and the back titrations are *x* for ammonia plus urea, and *y* for urea alone, then

$$B - y = k_1 CO(NH_2)_2,$$

$$y - x = k_2 NH_3.$$

The empirical values of k_1 and k_2 are determined for each lot of solutions and each sample of permutit and are controlled from time to time during experiments against pure solutions of ammonium sulphate and of urea and also of mixtures of the two. The method was used for quantities of 1–20 μg. of each in 10 ml. of water. Each determination took about 45 min.

A determination was made as follows: two samples of water, one before the eggs or alevins had been in many minutes, and a second after a recorded period of time. A sample of plain tap water was analysed also—it served as a blank control on the reagents used. The number of individuals was noted in each case. The interpretation of results of analyses was difficult when eggs died during a determination or when the alevins hatched or suffered damage to the yolk-sac. When deaths occurred numbers were ambiguous and the amino fraction was invariably increased by diffusion through the dead egg membrane of products not released by the living egg. Hatching liberated the syrupy vitelline liquid analysis of which therefore gave similarly enhanced amino-nitrogen values. Escape of yolk from the sac by damage leads to its denaturation and affects the amino-nitrogen values similarly. Two samples of 50 ml. of the water sample and of 50 ml. of tap water for blank are measured into 100 ml. graduated flasks. To each 20 ml. *N*/100 hydrochloric acid is added so that the resulting solution is very slightly acid (roughly 0·2 ml. 0·01 *N* HCl in excess). To one sample 1 g. of permutit is added and shaken for 5 min. The flasks are made up to 100 ml. with ammonia-free glass-distilled water.

The procedure in titration is to withdraw 10 ml. samples into carefully cleaned 50 ml. Kavalier Erlenmeyer conical flasks (this glass is not tinted) covered with a glass cap. 2 ml. of the sodium hypobromite solution is added and the cover replaced, then shaken and left for 3 min. (measured by stop-watch), 0·5 ml. 5% hydrobromic acid is added and after 30 sec. naphthyl red solution is run in from a microburette until the pink colour of the dyestuff persists. The composition of the naphthyl red solution and the sodium hypobromite solution are given by Teorell (1932).

It is essential that the flasks be clean, and the several stages of the titration must be performed to fixed times as the volume of the naphthyl red required depends upon:

(1) the duration of the titration;
(2) the interval between the addition of hypobromite solution and acidification;
(3) the interval between acidification and titration.

(*e*) *The determination of total nitrogen content*

Determinations of the total nitrogen in the dried samples of embryo and yolk were made by a microchemical modification of Kjeldahl's method. Errors arising from limitations of technique lay within $\pm 0{\cdot}5\%$ for determinations using between 2–6 mg. of ammonium sulphate. Analytical fluctuations beyond this range on the trout material may be attributed to lack of homogeneity in the samples.

(*f*) *Determination of carbohydrate*

Details of the analytical procedure used in determining the total carbohydrate content of egg embryo and yolk-sac appear in the next paper of the series. For the argument of the present communication it is sufficient to state that the amount of carbohydrate present at any time and consumed during certain limited periods is so small (143 cal./100) in proportion to the total protein and fat consumption, some 10,000 cal., that in the general exposition of the balance sheet the carbohydrate content may be neglected.

IV. EXPERIMENTAL RESULTS

(*a*) *The wet and dry weights of eggs, embryo and yolk*

Table 1 shows the wet weights in grams per 100 of egg, alevin and embryo and the yolk values calculated by difference. A study of these figures reveals no significant change in egg weight from fertilization to hatching. Until about the 40th day embryo growth proceeds slowly. The rate of weight increases slackens temporarily around the 50th day after which the rate of growth increases again until the 73rd day when a decline attributable to the using up of the yolk is observed. The alevin reaches its maximum weight before the embryo, a confirmation of Gray (1926). The wet weight of the whole increases as the intake of water by the system exceeds the loss in dry material resulting from catabolism. In the egg the restriction of water intake by the shell ('chorion') is a feature of development although Krogh and Ussing (1937) have observed that penetration of heavy water through the chorion is possible at all times and through the vitelline membrane after the eggs become 'eyed', some 15 days incubation at 10° C. The dry weights of 100 embryos and 100 yolks at various ages are also shown in Table I. The figure for the total weight of the system are obtained by adding the weight of the embryo and yolk respectively. This total decreases throughout the period studied, with a continuously accelerated slope corresponding to the increasing metabolic needs of the growing fish.

(*b*) *Heat production of the egg and alevin*

The measured rates of heat production expressed in calories produced by 100 individuals in an hour for the light batch of eggs of mean weight 90·0 mg. (1936–7) are given in Table 1 and Fig. 1. Up to the 34th day eggs were used for the determination; after this, alevins. The number used varied from 100, when the anticipated

Table 1. *Wet and dry weights of egg, embryo and alevin*

Age	G. wet weight/100 individuals			G. dry weight/100 individuals		
	Alevin	Embryo	Yolk	Alevin	Embryo	Yolk
1	8·659	—	—	2·999	—	—
1*U*	9·078	—	—	3·141	—	—
22	—	0·368	—	—	0·048	—
27	—	0·572	—	—	0·092	—
32	8·76	0·872	7·89	3·136	0·108	2·995
32*m*	—	1·469	—	—	0·173	—
35	8·199	1·183	7·016	2·935	0·146	2·789
41	8·257	1·620	6·637	2·891	0·199	2·692
44	8·622	2·421	6·201	2·843	0·289	2·554
47	9·110	3·225	5·886	2·833	0·392	2·441
50	9·439	3·853	5·586	2·800	0·490	2·309
53	10·213	5·137	5·076	2·656	0·657	1·999
56	10·898	7·101	3·797	2·559	1·025	1·534
62	12·028	9·615	2·413	2·612	1·314	1·298
65	12·420	10·332	2·088	2·266	1·547	0·719
71	12·338	11·371	0·967	2·069	1·814	0·255
77	11·796	11·429	0·367	1·868	1·786	0·082
83	—	10·683	—	—	1·667	—

Notes. *U* = sample of unfertilized eggs from the same batch. *m* = the embryo sample includes the embryonic membrane (yolk-sac).

Table 2. *Heat production measurements*

Age	Cal./100/hr.	Age	Cal./100/hr.	Age	Cal./100/hr.	Age	Cal./100 hr.
1–2	−0·214	23	1·07	45	3·40	63	10·32
2–3	+0·13, −0·086	25	0·98, 0·50	47	3·28	65	13·19
3	−0·38	27	0·50, 0·89	49	4·23	66	11·85
4	+0·83, +0·72	29	0·35	51	5·49	68	10·34
6	0·48, 0·46	31	0·53, 0·91	53	5·92	69	5·72
8	0·15, −0·5	33	0·58	55	7·10	71	6·32
10	0·41	35	0·62	56	8·88	73	11·72
13	0·70, 0·58	36	1·28	57	8·06	74	14·72
14	1·02, 0·41	37	1·30	58	9·05	76	11·6
17	0·398, 0·82	39	1·51, 2·05	59	7·93	78	11·4
19	0·76, 0·53	41	2·53	60	9·54	80	11·58
21	0·96	43	2·26	62	10·36	83	9·19
22	0·73, 0·53						

heat production was low, to 25, so that irregularities due to population variability are not constant throughout the series. Figures for the heat production before the 15th day are only to be accepted with reserve. At this time several eggs died during each determination (up to a maximum of 80% between the 5th and 12th days), and it was difficult in the earliest stages to determine in the living egg how many were fertile. Not until the egg is 'eyed' is it possible to ascertain fertility with certainty.

Within 24 hr. of fertilization heat production is negligible. Gastrulation overgrowth begins on the 4th–5th day and proceeds until the 11th day under the

conditions of incubation. The peak of heat production on the 4th day would then correspond to the end of segmentation. For short periods (5th–10th day) and before hatching at the 35th day the rate of heat production declines. It should be noted that until hatching was finished the agreement between duplicate measurements was less good than was to be expected from the physical characteristics of the calorimeters. As soon as the alevin hatched a marked increase in the rate of heat production was observed: this coincides with a marked increase in the rate of embryonic growth. The considerable fluctuations in heat production observed between the 65th and 83rd days have been confirmed during three seasons of observations.

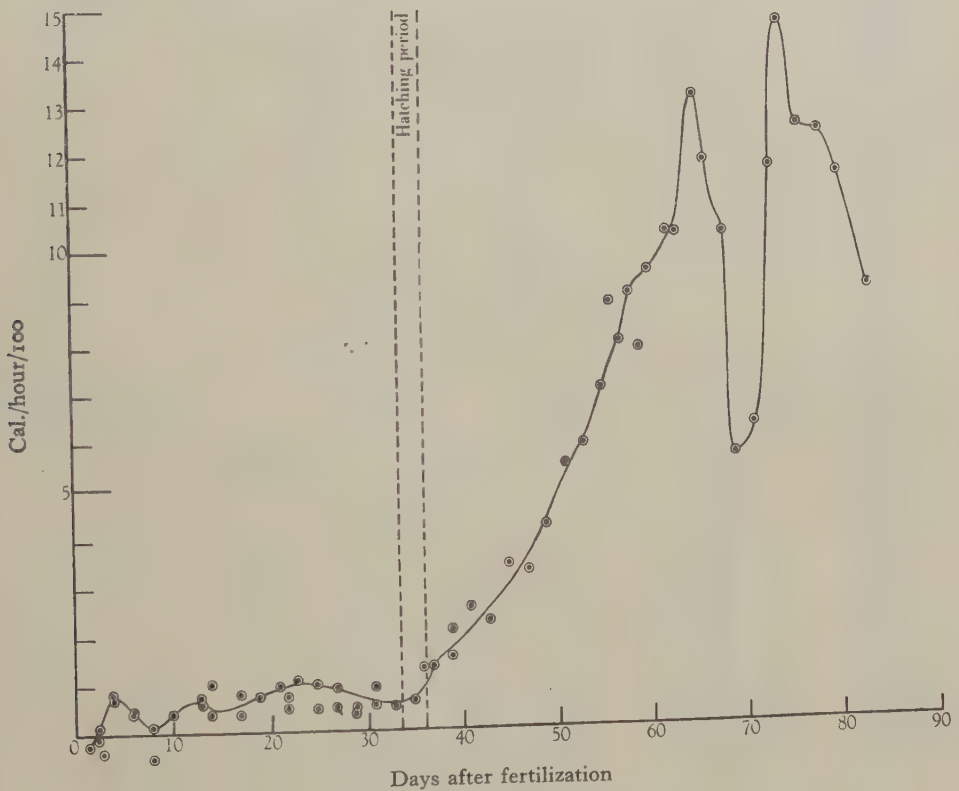

Fig. 1. Rate of heat production in calories per hour for 100 eggs or alevins throughout development.

(*c*) *Fuel values of eggs, embryo and yolk*

The fuel values of egg, embryo, and yolk are given in Table 3. The samples used for these determinations were from a larger batch of eggs incubated for the purpose, on which heat production observations were not made. The mean weight of these eggs (66 mg.) was smaller than that of the 1936–7 batch (90 mg.), and the length of the yolk-sac period was shorter by some 3 days in 70. The time scale of development is influenced by size of egg and accordingly these fuel values were applied to

the 1936–7 measurements by relating corresponding stages in the growth process instead of corresponding days of incubation. The acceleration in growth rate after hatching and the peak of embryo growth were selected for this purpose. The fuel values are used to calculate the total fuel value of the embryo, yolk and alevin in Table 4. Columns 2 and 4 show the dry weights of yolk and embryo respectively; column 5 the fuel value of 100 embryos, and column 3 the fuel values of the yolk

Table 3. *Fuel values of embryo and yolk*

Age	Calorific value per g. dry embryo	Calorific value per g. dry yolk
1–2	—	6·035
41	4·271 (pooled sample of three ages)	6·242
45		6·202
48		6·184
53	5·248	6·389
56	—	6·034
65	5·190	5·935
69	5·471	—
73	5·145	—
77	5·242	—
81	5·001	—
90	5·095	—

Table 4. *Calorie balance sheet*

Age	Dry wt. of yolk (g./100)	Fuel value of yolk (cal./g. dry wt.)	Dry wt. of embryo (g./100)	Fuel value of embryo (cal./g. dry wt.)	Heat content cal./100			Total heat production	Estimated heat energy of the system in calories
					Embryo	Yolk	Alevins		
1	2	3	4	5	6	7	8	9	10
36	2·773	6,246	0·153	4,270	653	17,300	17,953	0	17,953
39	2·736	6,210	0·179	4,540	812	17,000	17,812	108	17,920
42	2·648	6,180	0·220	4,730	1,041	16,400	17,441	249	17,680
45	2·525	6,160	0·326	4,940	1,610	15,600	17,210	444	17,654
48	2·398	6,150	0·458	5,140	2,354	14,770	17,124	692	17,816
51	2·230	6,120	0·620	5,293	3,282	13,640	16,922	1,027	17,949
54	1·840	6,080	0·797	5,390	4,296	11,200	15,496	1,449	16,945
57	1·540	6,040	1·068	5,450	5,821	9,310	15,130	2,009	17,140
60	1·286	6,020	1·243	5,420	6,780	7·750	14,530	2,670	17,200
63	0·992	5,980	1·434	5,380	7,772	5,940	13,712	3,439	17,151
66	0·607	5,920	1·624	5,336	8,747	3,590	12,337	4,339	16,676
69	0·388	5,910	1·760	5,290	9,431	2,295	11,726	5,014	16,740
72	0·223	5,910	1·821	5,240	9,633	1,320	10,953	5,495	16,448
75	0·128	5,910	1·818	5,188	9,526	758	10,284	6,714	16,998
78	0·062	5,910	1·768	5,137	9,172	370	9,542	7,567	17,139
81	0·010	5,910	1·707	5,102	8,770	180	8,950	8,372	17,322
84	0·000	—	1·630	5,102	9,316	—	8,316	9,055	17,371

at the same stage. The heat content of embryo and yolk are given in columns 6 and 7 respectively, the total heat content of 100 alevins is the sum of these, and is given in column 8.

(*d*) *Energy balance throughout development*

The heat produced up to a given stage in development was calculated by numerical integration from the heat-production measurements. The values obtained in this way are shown in column 9 (Table 4).

The sum of columns 8 and 9 (Table 4) should be constant within the limits of the errors of determination of the quantities concerned if the first law is applicable to embryo systems. The disparity between the heat production observed and that to

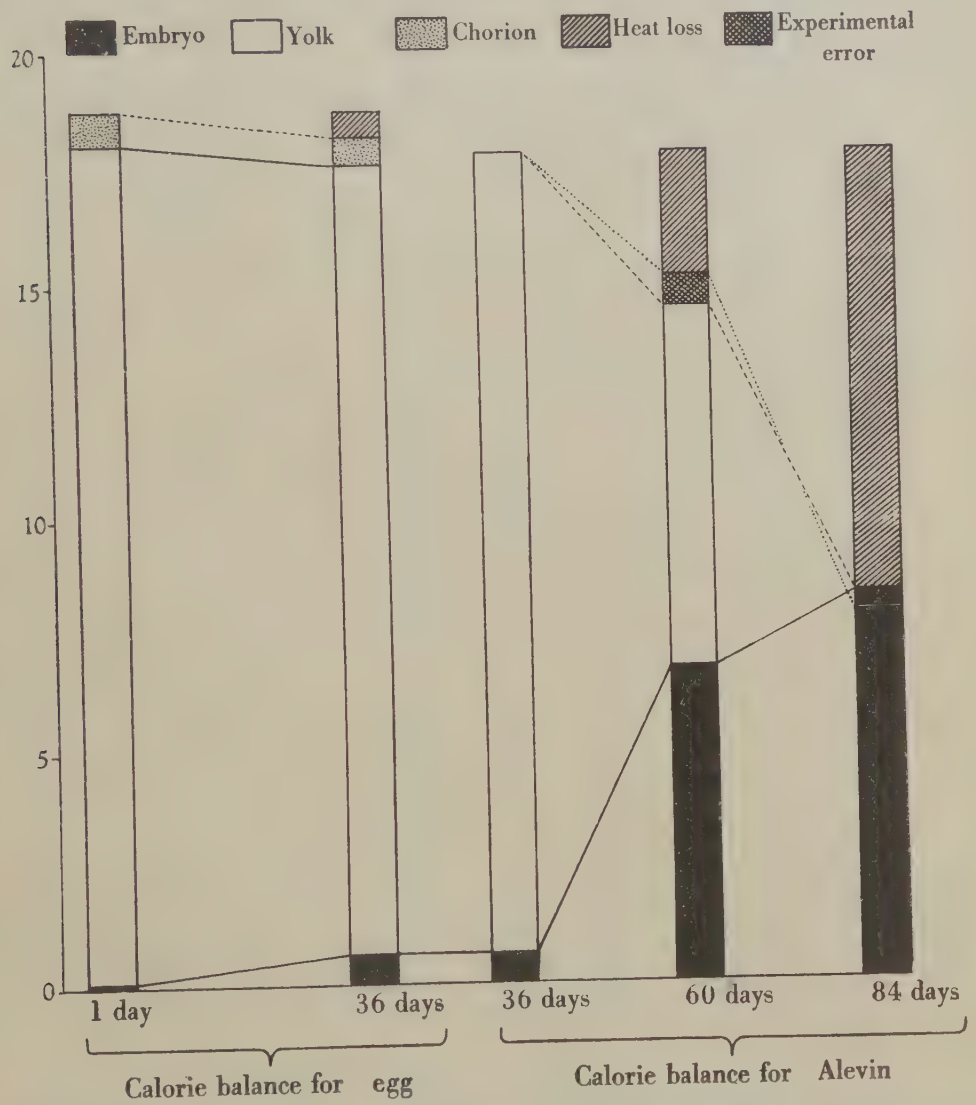

Fig. 2. Diagram to illustrate the balance sheet for heat production and fuel values between the fertilized egg and hatching, and over the period of life of the free living alevin. (Amounts are given in kilocalories and refer to 100 individuals.)

be anticipated from the fuel determinations is shown in Fig. 2 where the balance for the egg between fertilization and hatching and the beginning, middle, and end of the yolk-sac periods are represented schematically. The increase in integrated total heat evolved in development is shown by the increase in length of the upper section of

the column; the fuel value of the yolk is shown in the middle unit and that of the embryo in the lowest part. The discrepant parts of the balance are indicated by the hatched or shaded portions, these latter represent a fraction within the limits of error of the techniques employed and it is therefore reasonable to assume that the first law of thermodynamics is valid for the development of the trout embryo from egg to young fish.

Table 5. *Experimental figures for the nitrogen excretion**

Age	Ammonia per 100/hr. expressed as μg. of N		Urea per 100/hr. expressed as μg. of N		Total nitrogen μg./100/hr.		Percentage of nitrogen excreted as ammonia	
21	3·86		7·14		11·00		35·0	
23	9·04		1·82		10·86		83·2	
25	7·79		0·45		8·24		94·5	
27	8·29		1·05		9·34		88·7	
29	7·95		6·02		13·97		—	
31	16·7		50·3		67·0		—	
33	10·35		7·61		17·96		57·8	
35	21·0	15·9	13·4	30·9	34·4	46·8	61·0	33·9
37	15·8		28·2		43·9		35·9	
41	23·6	35·2	4·97	9·94	28·6	45·1	82·7	78·2
43	28·2	22·1	7·7	6·53	35·9	28·6	78·6	77·3
45	20·2	23·5	11·7	14·6	31·9	38·1	63·3	61·7
47	19·3	16·9	14·2	15·0	33·5	31·9	57·7	53·0
49	23·4	31·5	10·6	14·6	34·0	46·1	68·8	68·4
51	27·2	34·5	17·6	14·6	44·8	49·1	60·7	70·3
52	47·7		23·0		70·7		67·5	
53	39·9	42·5	12·1	14·3	52·0	56·8	76·8	74·8
55	29·7	48·8	27·7	27·9	57·4	76·7	51·7	63·7
56	63·2		19·5		82·7		76·5	
57	52·9	53·2	19·0	35·0	71·9	88·2	73·6	60·2
58	61·2		26·6		87·8		69·6	
59	58·8		26·6		85·4		69·0	
60	55·3		32·1		87·4		63·4	
62	41·7	60·9	64·4	26·6	106·1	87·5	39·3	69·5
63	69·2		25·5		94·7		73·1	
65	68·8	55·8	35·4	49·0	104·2	104·8	66·0	53·4
66	45·6		80·1		125·7		36·3	
68	64·7	57·4	42·9	49·0	107·6	106·4	60·2	53·0
69	79·8		27·4		107·2		74·5	
71	63·6	61·5	63·4	42·9	127·0	104·4	50·2	58·9
73	74·8	37·6	36·9	65·2	111·7	102·8	64·0	36·6
74	44·8		75·5		120·3		37·3	
76	42·8	45·8	57·9	68·5	111·3	103·7	38·6	44·3
78	95·0	73·6	58·2	51·2	153·2	124·8	61·7	59·0
80	64·4	55·0	61·7	53·7	126·1	108·7	51·0	50·7
83	74·5	62·2	56·9	74·6	131·4	136·8	56·7	45·4

(*e*) *The nitrogen excretion of the egg and alevin and the total nitrogen content*

The amount of ammonia and urea nitrogen excreted by the trout and alevin are given in Table 5; daily rate of nitrogen excretion is shown in Fig. 3. The hourly excretion of each 100 individuals rises, with short periods of arrested rates of increase at the 28th–29th, 37th–47th and 58th–60th days. From the 75th day onwards there is a wide variability in rate of excretion and no general trend is discernible. The

* The figures on the right-hand sides of columns 2–5 refer to duplicate determinations of the corresponding figures on the left-hand sides of the columns.

percentage of ammonia excreted by the egg is very high and there is no certain evidence of urea excretion by diffusion through the chorion before hatching. The slightest injury to an egg leads to the appearance of amino-nitrogen in the circumambient water. The few occasions on which urea excretion was negligible thus are taken to indicate the more probable state of affairs. The perivitelline fluid released on hatching contains large amounts of amino-nitrogen which seems unable to diffuse through the chorion. The perivitelline fluid of each egg produces on the average 37 μg. urea nitrogen. This may have accumulated as a product of the

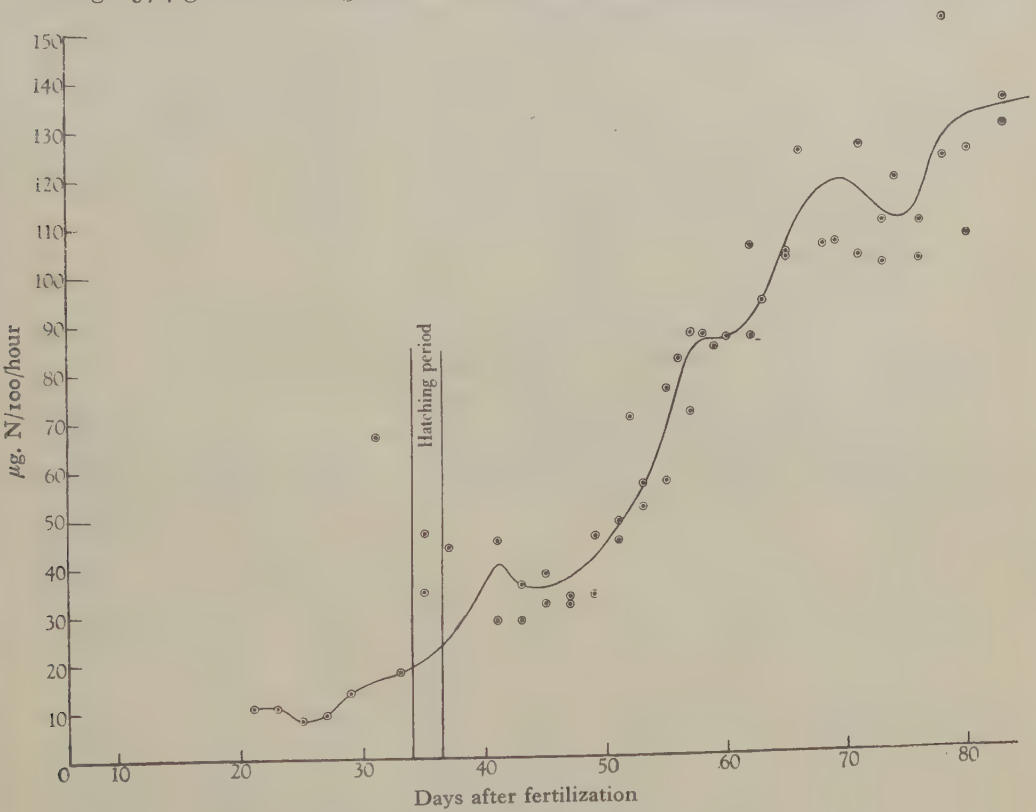

Fig. 3. Rate of nitrogen excreted in μg. per hour for 100 eggs or alevins throughout development.

embryonic metabolism or as a result of digestion of the chorion by a hatching enzyme (Wintrebert, 1912) secreted by specialized cells in the ectoderm of the embryo.

The results of the total nitrogen analysis by micro-Kjeldahl methods are given in Table 6. The percentage of nitrogen in the yolk fraction changes considerably towards the end of the yolk-sac period, which changes may be attributed to selective absorption of proteins and to a proportionate increase of the remaining yolk fat. From this cause a decline in percentage nitrogen content occurs between the 45th and 70th days. The slight fluctuations in the embryo percentage nitrogen content

while of statistical significance are not easy to interpret. The decline from the 75th day arises from the combustion of muscle protein by the starving alevin.

(*f*) *The nitrogen balance*

Table 7 was compiled using the results of dry-weight determinations for the development as an alevin from Table 1 and the percentage nitrogen figures of Table 6. Column 1 gives the age; 2 and 3 the figures to permit the calculation of

Table 6. *Percentage of nitrogen in the dry samples of embryo and yolk*

Age	Embryo	Yolk	Age	Embryo	Yolk
2	—	10·31	59	12·04	9·63
29	11·62	10·44	62	11·61	9·71
34	11·83	10·42	65	11·52	9·15
36	11·68	10·46	68	11·87	7·42
38	11·54	10·54	71	11·65	7·43
41	11·73	10·56	74	11·75	6·84
44	11·93	10·49	77	11·80	7·94
47	11·73	10·13	80	11·43	—
50	12·11	10·57	83	11·57	—
53	12·09	9·94	86	11·16	—
56	11·87	10·08			

Table 7. *Nitrogen balance sheet*

Age	Dry weight of yolk g./100	Percentage nitrogen yolk sample	Dry weight of embryo g./100	Percentage nitrogen embryo sample	Total nitrogen in 100 embryos	Total nitrogen in 100 alevins	Total nitrogen excreted	Estimate of the total nitrogen in the system
1	2	3	4	5	6	7	8	9
36	2·773	10·50	0·153	11·74	0·0180	0·3092	0·0000	0·3092
39	2·736	10·52	0·179	11·82	0·0212	0·3090	0·0032	0·3122
42	2·648	10·51	0·220	11·86	0·0261	0·3045	0·0064	0·3109
45	2·525	10·48	0·326	11·89	0·0388	0·3035	0·0091	0·3126
48	2·398	10·37	0·458	11·96	0·0549	0·3035	0·0121	0·3156
51	2·230	10·26	0·620	12·01	0·0745	0·3035	0·0156	0·3191
54	1·840	10·12	0·797	12·01	0·0947	0·2818	0·0196	0·3014
57	1·540	9·93	1·068	11·97	0·1278	0·2806	0·0252	0·3058
60	1·286	9·69	1·243	11·94	0·1483	0·2728	0·0315	0·3043
63	0·992	9·19	1·434	11·88	0·1705	0·2617	0·0382	0·2999
66	0·607	8·48	1·624	11·83	0·1920	0·2435	0·0462	0·2897
69	0·388	7·71	1·760	11·77	0·2073	0·2372	0·0552	0·2924
72	0·223	6·98	1·821	11·72	0·2136	0·2292	0·0643	0·2935
75	0·128	7·07	1·818	11·63	0·2115	0·2205	0·0725	0·2830
78	0·062	8·30	1·768	11·56	0·2046	0·2097	0·0813	0·2900
81	0·010	8·30	1·707	11·46	0·1958	0·1966	0·0917	0·2883
84	—	—	1·630	11·35	0·1850	0·1890	0·1015	0·2910

nitrogen in the yolk-sac; and 4 and 5 similar figures for the embryo. The total nitrogen for the alevin is given in column 7, while column 8 gives the results of the total nitrogen excreted calculated by Simpson's rule from the smoothed curve of total nitrogen excretion (Fig. 3). Column 9 is the sum of columns 7 and 8, and this total represents the total of nitrogen in the alevin system and of nitrogen lost by the system; this sum should be constant. The range of variation

observed in column 9 affords a check upon the accuracy of the experimental techniques employed. Up to the 35th day a computation of the nitrogen balance sheet is difficult as only ammonia nitrogen is lost before hatching. This process entails the loss of urea nitrogen as the perivitelline fluid is released, while at the same time the material of the chorion has been dissolved by the hatching enzymes and the products of solution are absorbed by the alevin in considerable amount. An analysis of the chorions remaining after hatching was not made. These were difficult to collect and the viscid scum which they formed did not permit an estimate of their number. Careful analyses of the chorion were made some days before this, when it is a tough thick membrane; after hatching it is thin and delicate. A sequence of weight readings from another egg batch when a number of chorions was allowed to dry to constant weight in air gave the following figures which are consistent between themselves being all from the same batch of eggs.

Age	100 chorions
28	0·268 g.
29	0·274 g.
34	0·170 g.

Hatching occurred at 34 to 36 days with this sample.

There is no doubt that a considerable solution of chorion occurs at hatching, and the evidence shows that such products of solution are absorbed to a considerable extent by the alevin system. From Tangl & Farkas' (1904) figures 100 eggs contained 0·359 g. nitrogen while after hatching 100 alevins contained 0·357 g. nitrogen. Losses from the chorion were thus negligible. The balance between fertilized egg and hatched alevin are:

Nitrogen:	At 2 days		At 36 days	
	In embryo and yolk	0·3041 g.	Embryo and yolk	0·3092 g.
	In chorion	0·0186 g.	Total excretion through shell	0·0072 g.
			Total hatching excretion loss	0·0037 g.
		0·3227 g.		0·3201 g.

(The chorion figure in the left-hand column is calculated from Hayes's (1930) figure of percentage nitrogen using his dry weight as a basis of comparison.) Throughout development the nitrogen of the egg may be considered accounted for, if due allowance be made for errors of experiment.

V. ANALYSIS OF EXPERIMENTAL RESULTS

The correlation of weight loss with heat production and nitrogen excretion

The substantial agreement between analyses of dry material and the observations on the living material, shown in the calories and nitrogen balances, invites some attempt to correlate these two sets of observations with the observed weight losses of the system. The procedure and argument are summarized in Table 8 and they are as follows:

The figure for cumulative nitrogen excretion in column 2 (up to the age given in

Table 8

Age	Total nitrogen excretion	Total calories of heat produced calculated from the nitrogen excretion	Total calories of heat measured	Calories of heat not produced from protein break-down assumed fat	Weight of fat lost to system calculated from previous column	Weight of protein loss calculated from col. 2	Total estimated loss in weight (from the meta-bolism) cols. 6 and 7	Residues (see text)	Differences from the mean residue [0·115 g./100]	Observed total dry weight loss since fertilization	Observed dry weight of the alevin
1	2	3	4	5	6	7	8	9	10	11	12
36	0·0072	189	598	409	0·044	0·045	0·089	0·105	−0·010	0·194	2·926
39	0·0104	273	706	433	0·046	0·065	0·111	0·095	−0·020	0·206	2·915
42	0·0136	358	847	489	0·052	0·085	0·137	0·116	+0·001	0·253	2·868
45	0·0163	428	1042	614	0·065	0·102	0·167	0·103	−0·012	0·270	2·851
48	0·0193	507	1290	783	0·083	0·121	0·204	0·061	−0·054	0·265	2·856
51	0·0228	599	1625	1026	0·109	0·143	0·252	0·019	−0·096	0·271	2·850
54	0·0268	704	2047	1343	0·143	0·168	0·311	0·173	+0·058	0·484	2·637
57	0·0324	852	2607	1755	0·187	0·202	0·389	0·121	+0·009	0·510	2·608
60	0·0387	1017	3268	2251	0·239	0·242	0·481	0·111	−0·004	0·592	2·529
63	0·0454	1190	4037	2847	0·303	0·284	0·587	0·109	−0·006	0·696	2·426
66	0·0534	1402	4937	3535	0·376	0·334	0·710	0·180	+0·065	0·890	2·231
69	0·0624	1640	5612	3972	0·422	0·390	0·812	0·161	+0·046	0·973	2·148
72	0·0715	1880	6093	4213	0·447	0·447	0·894	0·183	+0·068	1·077	2·044
75	0·0797	2090	7312	5222	0·555	0·498	1·053	0·122	+0·007	1·175	1·946
78	0·0885	2320	8195	5875	0·666	0·554	1·220	0·071	−0·044	1·291	1·830
81	0·0989	2600	8970	6370	0·677	0·618	1·295	0·109	−0·006	1·404	1·717
84	0·1087	2860	9653	6793	0·723	0·680	1·403	0·09	−0·025	1·49	1·63

All figures expressed in g./100 individuals or in cal./100 individuals.

column 1) are converted into estimated weight loss assuming the nitrogen excreted to be derived from protein catabolism and that the factor $6{\cdot}25 \times N$ obtains for embryonic systems. The loss during hatching and the estimated nitrogen content

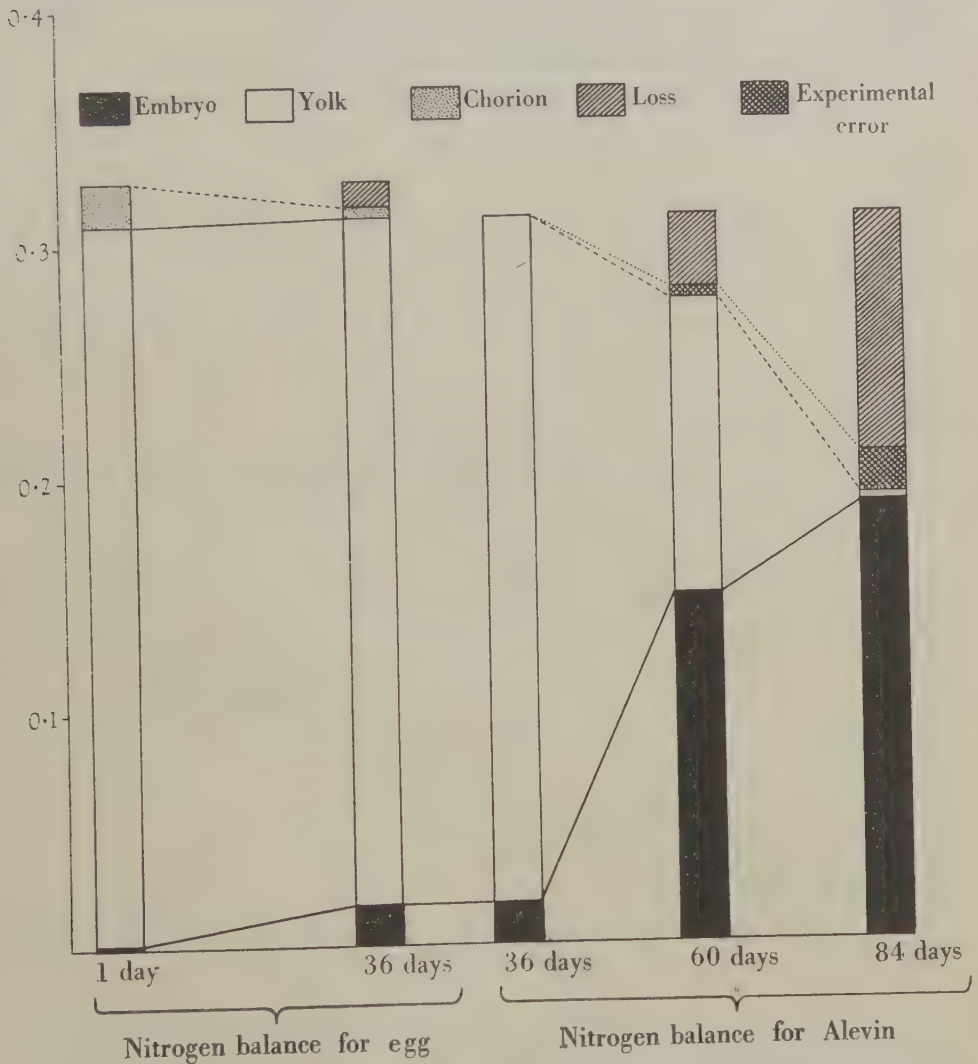

Fig. 4. Diagram to illustrate the balance sheet for nitrogen excretion and nitrogen content between the fertilized egg and hatching and for the period of free life of the alevin. (Amounts are given in g. of nitrogen and refer to 100 individuals.)

of the chorion is omitted from this series of figures. These weight losses are reproduced in column 7 of the table. They are converted into estimated heat losses (column 3), by multiplying with the factor 4200 which is an average estimate of the fuel value of protein. The integrated cumulative heat losses of the system are given in column 4. Some part of each heat-loss figure may be ascribed to protein com-

bustion, the carbohydrate contribution is small and may be neglected and the difference column 4 minus column 3 therefore may be taken to represent the heat energy lost by the system through the combustion of fats. Column 5 gives such estimated figures. These may be converted into weight losses by dividing each figure by 9400 which is a typical fuel value for fat. The fat weight losses are given in column 6, and the protein weight losses in column 7. The total loss from fat and protein combustion is seen in column 8, being the sum of 6+7.

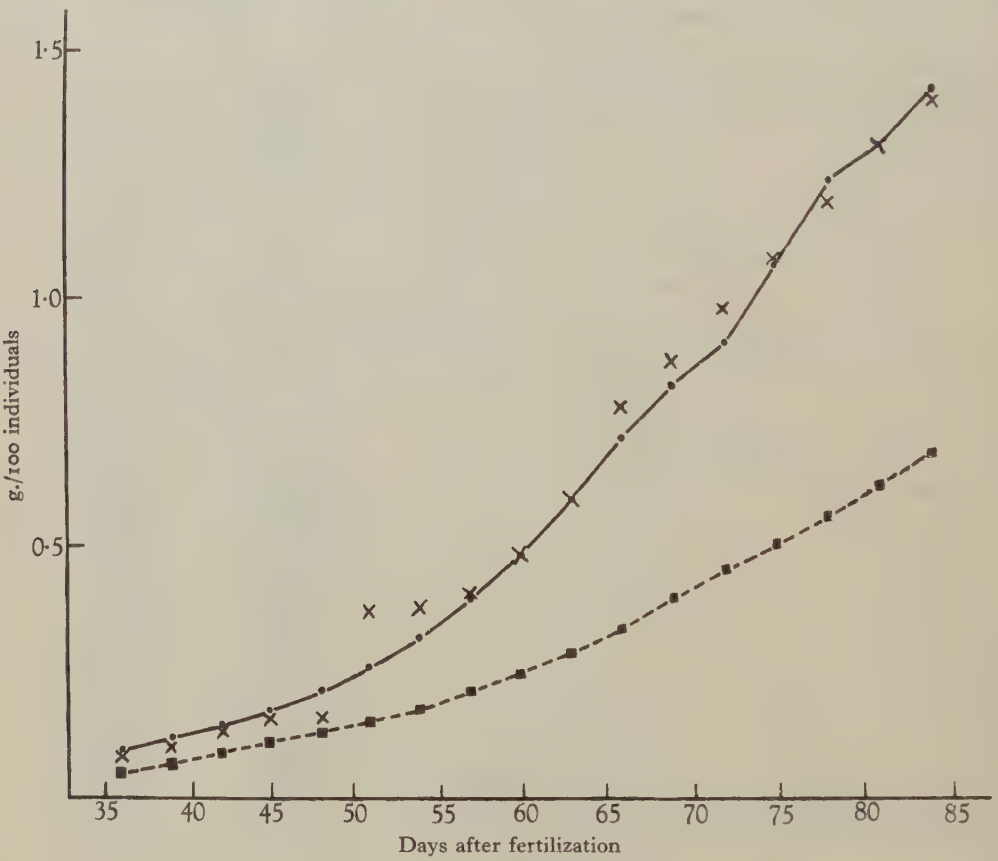

Fig. 5. Graph to show the relationship between the observed dry-weight losses of the alevin and the losses inferred from the nitrogen excretion observed and the heat production. ■ = protein loss; ● = total of fat + protein loss; × = dry-weight loss.

On the right-hand side of the table the dry weights of the alevins are given in column 12. The dry weight of the eggs is 3·121 g./100. Column 11 supplies the differences between this value and the dry weights of column 12 and each figure accordingly represents the observed loss in weight of the system. This of course includes the chorion and weight of the nitrogen substance released on hatching. Subtracting from this observed weight loss (column 11) the estimated metabolic losses of column 8, the 'residues' given in column 9 result. This residue is seen to

fluctuate around a mean value of 0·115 g./100. This is a reasonable figure for the solids lost at hatching, which include the chorion residue and some of the products of solution of the chorion.

If all losses were correctly traced to metabolism and to chorion loss on hatching all these residues in column 9 should be constant, their fluctuation from the mean 0·115 is given as a difference in column 10. These differences are inconsiderable in amount compared with the possible errors in the dry-weight determinations, it is therefore reasonable to assume all weight losses in the system accounted for as loss in chorion at hatching and as fat and protein catabolized in the maintenance of the growing system. Fig. 5 expresses the results of this correlation, the lower curve gives the estimated cumulative loss in weight from protein catabolism, the upper the sum of the protein and fat losses. The crosses are the dry-weight losses less 0·115 hatching loss. There is clearly no significant difference between the curve deduced from the metabolism and the figures for dry-weight loss.

VI. DISCUSSION

This paper presents the results of an attempt to observe, as completely and directly as possible, the energy changes occurring during embryonic development in the trout. It has been shown that the dry-weight losses of the system are in accordance with those calculated from the results of simultaneous measurement of heat production and of protein breakdown. A study of the carbohydrate metabolism to be described in a later paper has shown that for present purposes the energy changes associated with this fraction of the total metabolism may be neglected. It was found that the standard caloric equivalents for normal adult metabolism were also valid for the developing trout in respect to both protein and fat. There is therefore no essential difference between the thermal efficiency of embryonic and adult metabolism, within the limits of experimental error of the procedures employed. It is also clear that the first law of thermodynamics applies to embryonic development.

In the course of this analysis separate balance sheets for the heat energy of the system and its progressive dissipation, and for the total nitrogen of the system have been compiled. These show that all the nitrogen in the egg after fertilization is either excreted or converted into embryo. Similarly the total fuel energy of the egg is either dissipated or stored in the embryo. It seems relevant at this juncture to examine previous work within this field.

The most important work is that of Bohr & Hasselbalch (1902) on the metabolism of the hen's egg. They made simultaneous measurements of the heat production and carbon dioxide output of single eggs, but were not able to make observations on a single egg throughout the entire period of incubation. From their results for different eggs of different ages they compile a graph which shows that the heat production observed corresponds to the heat production expected on theoretical grounds from the carbon dioxide production within an error of 4%. They assumed that all the carbon dioxide was derived from the metabolism of fats and that the normal calorie equivalent for fats applied. Though the early days of incubation were not studied in detail this work is the most considerable study yet published.

Gayda (1921) made extended measurements of the heat production of embryo and larval toads (*Bufo vulgaris*). He did not measure respiration nor did he perform chemical analyses. The heat production observed was correlated with empirical estimates of the surface area. For much of the period studied the larvae were feeding and living a normal active life. It was thus impossible to draw up a balance sheet relating energy intake to growth and to heat production. Gayda's results were subjected to mathematical treatment by Wetzel (1937), for comment on this work Needham (1942, § 3·21) may be consulted.

In attempting to compile a balance sheet for the energy changes during development from his own and Murray's studies of the chemical embryology of the chick, Needham (1931) experienced great difficulty in adjusting the results of several independent workers. His conclusion that: 'it may, therefore, be assumed as probable that the true calorific constants for the substances present in early embryonic life (in the case of the chick) should be regarded as lower than those for the corresponding adult substances' certainly does not apply to the development of the trout.

Until recently it was supposed that skeletal and muscular systems were permanent machinery which, though requiring periodic repair, did not need continual substantial replacement, and that a part of the energy requirement of growth was devoted to the establishment of organized systems of this kind. From the work on the rate of exchange of chemical molecules (using radioactive isotopes) (summarized in Rittenberg, 1941) in the organized tissue systems of the adult mammal, it now seems certain that a fairly rapid turnover of the constituent amino-acids of a muscle protein, for instance, occurs. Maintenance thus becomes of far greater importance than was envisaged by earlier workers on the energy relations of development.

Since orderliness can only be expressed in physical terms by invoking the concept of entropy (see Schrödinger, 1944), there has been a tendency in discussing the development of structural pattern in living things, to suppose that they evade in some way the limitations of physical laws. A developing organism, increasing in structural complexity, appears at first sight, to be an impertinent exception to that great generalization 'Die Entropie der Welt strebt einem Maximum zu'. What is forgotten is that the entropy of the system as a whole is increasing as a result of the normal maintenance of living tissue and the exothermic breakdown of food materials to provide in sufficient amount the specific substrates necessary for development and growth.

A study of the energy balance between two arbitrary states, let us say the beginning and the end of embryonic development, can furnish no measure of entropy changes in some restricted part of the system. A local decrease in entropy will be concealed by the nett entropy increase of the system as a whole. Moreover, it seems extremely probable that local decreases in entropy (associated with the formation of fibrillar structures of various kinds, for example Picken (1940)) are intimately related to local syntheses. The inadequacy of entropy considerations alone and the importance of Gibb's free energy has been pointed out by Butler (1946) who

concludes: 'if an organism can synthesize peptide bonds, it appears that it will have no great difficulty in putting together protein molecules of any degree of complication. The free energy must come from the metabolic processes going on in the organism.... There is thus no outstanding difficulty in accounting for the synthesis of living structures with a fairly modest expenditure of food.'

Until more is known of the energetics of chain molecule synthesis and fibrillar aggregation in biological systems we cannot expect to proceed further in the detailed study of the energetics of embryonic growth and development.

VII. SUMMARY

1. The wet and dry weights of a selected population of rainbow trout eggs were determined at intervals from the 1st day after fertilization until the 84th day of incubation at 10° C. The alevin attains its maximum weight at the 65th day, the embryo some 10 days later.

2. The heat production of egg and alevin rises until the 65th day and subsequently fluctuates violently. Fuel values of yolk and embryo samples were measured.

3. The heat production is commensurate with the loss in the total fuel value of the system.

4. Nitrogen excretion of eggs and alevin rises steadily throughout development. It seems probable that only the ammonia fraction is freely diffusible through the chorion.

5. Determinations of the total nitrogen content of embryo and yolk samples demonstrate that the nitrogen excretion observed is sufficient to balance the loss in total nitrogen of the system.

6. The fuel values and excretion measurements are shown to be in accord with the observed loss of dry materials by the egg-alevin system. The significance of this is discussed.

For the first two years of this investigation I received help from the Department of Scientific and Industrial Research. The Royal Society made two grants of money for apparatus. I was later a Research Fellow of St Catharine's College. For all such aid I am extremely grateful.

The stimulation, advice and interest of Prof. James Gray and Dr Laurence Picken have been indispensable. Without the support of the assistants in the department of zoology this work would have been beyond the powers of one person. I wish to thank them for such aid, especially D. R. Ashby and R. S. Undrill.

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TRANSPIRATION AND THE STRUCTURE OF THE EPICUTICLE IN TICKS

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(With Thirteen Text-figures)

The passage of water through the cuticle of living ticks is greatly influenced by the activities of the underlying epidermal cells. In a previous paper (Lees, 1946) it was shown, for example, that unfed ticks reach a state of equilibrium at a relative humidity of about 90%; and if their water balance is depleted they will take up water through the cuticle when exposed to higher humidities. Furthermore, although exposure to humidities below the equilibrium results in water loss through the cuticle, evaporation is much less rapid than it becomes subsequently if the tick is killed or asphyxiated. It was therefore argued that at low humidities secretion assists in the retention of water. These active processes are only well developed in the unfed tick and become imperceptible after the blood meal has been ingested.

In considering the mechanisms involved in the exchanges of water through the cuticle the assumption was made that, in addition to active secretion, the passage of water, and particularly its retention, is also influenced by the presence of lipoid material in the cuticle. Ticks show great diversity in their powers of resisting desiccation, and this was thought to be accounted for by the specific nature of the waterproofing lipoid. Nevertheless, no direct evidence of such a component was advanced in this paper.

Ramsay (1935), and more recently Wigglesworth (1945) and Beament (1945), have shown that the impermeability of insects is entirely due to a thin, discrete layer of wax or oil in the outermost part of the epicuticle. Any agents such as abrasive dusts, wax solvents or detergents, which interrupt the continuity of this layer, at the same time greatly increase transpiration. Water loss through the wax layer is also enormously increased if the temperature is raised above a certain critical value. In the present paper the methods devised by Wigglesworth for demonstrating the properties of the waterproofing layers in insects have been applied to a number of species of ticks (Acarina; Ixodoidea). The account also includes observations on the structure and deposition of the epicuticle, and on the functions of the dermal glands. The outermost layer of the tick cuticle visible in ordinary sections has hitherto been referred to as the 'tectostracum' (e.g. Ruser, 1933). As this paper will show, the similarity of this layer with the insect epicuticle is so marked that the abandonment of this term seems fully justified.

TEMPERATURE AND EVAPORATION FROM THE CUTICLE

Ramsay (1935) and Wigglesworth (1945) have shown that if whole insects with their spiracles covered are exposed for short periods to different constant temperatures, transpiration at first increases slowly as the temperature is raised, then much more abruptly when a certain temperature is exceeded. At this 'critical temperature', which varies widely in different insects, the waterproofing layer becomes more permeable to water (Beament, 1945).

Similar experiments have been carried out with a series of ticks of different species. The technique employed has already been described (Wigglesworth, 1945). Small batches of ticks, usually from two to six, were killed in ammonia vapour and their spiracles covered. They were then placed in a container of metal gauze which could be suspended over phosphorus pentoxide in a conical flask immersed up to the neck in a large water-bath. The temperature was then raised by intervals of about 10° C. and maintained constant for 30 min. at each temperature. After each exposure the ticks are weighed to determine water loss.

The rate of transpiration is expressed in mg./sq.cm. of surface/hr., and the results are therefore directly comparable with those of Wigglesworth. The surface areas of examples of each species have been estimated by cutting up the cuticle and either simply spreading the pieces on squared paper (engorged ticks) or by making measured drawings of the pieces with the aid of a camera lucida (unfed ticks). Since all the ticks were of approximately similar shape, two curves, one for unfed and the other for engorged ticks, could be constructed relating weight and surface area. Such a measure of evaporation implies that it is the apparent surface area and not the minute surface irregularities which, under the conditions of the experiment, are important in determining water loss. This assumption can easily be justified. During engorgement ixodids become enormously distended and the deep folds of the epicuticle are flattened out; nevertheless, there is, of course, no increase in the total surface area of the epicuticle, for this layer is completely inextensible. The relationship between apparent surface area and evaporation during engorgement is illustrated by the following example.

Six days after attachment unfed females of *Ixodes ricinus*, after removal from the host, had a mean surface area of 0·59 sq.cm. and lost water in dry air at 25° C. at the rate of 11·5 mg./sq.cm. during 24 hr. The average weight was 39·5 mg. Immediately after dropping from the host (8th day after attachment) the average weight was 311 mg., the surface area 1·87 sq.cm. and the rate of water loss 11·1 mg./sq.cm.

Results

The rates of water loss were first determined at a single temperature (25° C.) and with a series of living ticks representative of the families Argasidae and Ixodidae. The results obtained by exposing unfed and engorged ticks for 24 hr. to dry air are given in Table 1. The species form a graded series ranging from the least resistant member *I. ricinus* to the most resistant member *Ornithodorus delanoei acinus*. In the former species water loss is approximately fifty times more rapid than in the latter.

Ixodid ticks, it will be noted, are consistently less resistant than argasids, although species such as *Hyalomma savignyi* approach the degree of impermeability characteristic of the Argasidae.

Table 1 also shows that unfed ticks lose less water per unit of surface area than do the corresponding engorged stages. This difference, usually about fivefold, is too large to be explained by errors in determining surface area and is probably due to

Table 1. *Water loss from unfed and engorged ticks during 24 hr. in dry air at 25° C.*

Family	Species	Unfed		Engorged	
		mg./sq.cm.	% original weight	mg./sq.cm.	% original weight
Ixodidae	*Ixodes ricinus*	2·2	17·4	11·1	7·9
	I. hexagonus	1·8	8·1	6·4	4·8
	I. canisuga	1·3	8·7	3·3	3·7
	Amblyomma cajennense	0·76	2·6	2·0	1·3
	Dermacentor andersoni	0·26	1·3	1·7	1·0
	Hyalomma savignyi	0·24	0·8	1·3	0·4
Argasidae	*Ornithodorus moubata*	—	—	0·91	0·8
	O. parkeri	—	—	0·50	0·6
	Argas persicus	—	—	0·45	0·7
	Ornithodorus delanoei acinus	—	—	0·20	0·1

Table 2. *Approximate 'critical temperature' in ° C. (A), and temperature at which evaporation into dry air equals 5 mg./sq.cm./hr. (B)*

Family	Species	A	B
Ixodidae	*Ixodes ricinus*	32	34·5
	I. hexagonus	39	42·5
	Amblyomma americanum	41	44
	Haemaphysalis punctata	42	44
	Ixodes canisuga	42	46
	Amblyomma cajennense	43	46
	Dermacentor reticulatus	44	47
	D. andersoni	44	46·5
	D. variabilis	44	48
	Rhipicephalus sanguineus	45	47
	Hyalomma savignyi	45	48·5
Argasidae	*Ornithodorus moubata*	62	62
	O. parkeri	62·5	63
	Argas persicus	63	64
	Ornithodorus delanoei acinus	67	68
	O. savignyi	75	72

the ability of the unfed ticks to oppose evaporation by secretion. As was previously shown (Lees, 1946) the engorged tick usually lacks these powers. In the following experiments dead engorged ticks have been used almost exclusively.

The rates of transpiration in a series of ticks exposed to different temperatures are shown in Fig. 1. The approximate 'critical temperatures' and—as a more accurate standard of comparison—the temperature at which evaporation into dry air equals 5 mg./sq.cm./hr., have been read from the evaporation curves and are set out in Table 2.

All the species investigated fall into two distinct groups; moreover, what is particularly significant, the groupings correspond with the systematic position. In Ixodidae a sudden increase in transpiration takes place at a temperature which ranges from 32 to 45° C. In all Argasidae, on the other hand, the critical temperature is at least 15° C. higher and varies in different species from 62 to 75° C. The break in the evaporation curve is much less abrupt in the Argasidae, particularly in the higher members. Within each major group significant specific differences can be distinguished. In the Ixodidae there is a relatively compact subgroup including the more resistant species of *Ixodes*, and the genera *Amblyomma*, *Haemaphysalis*,

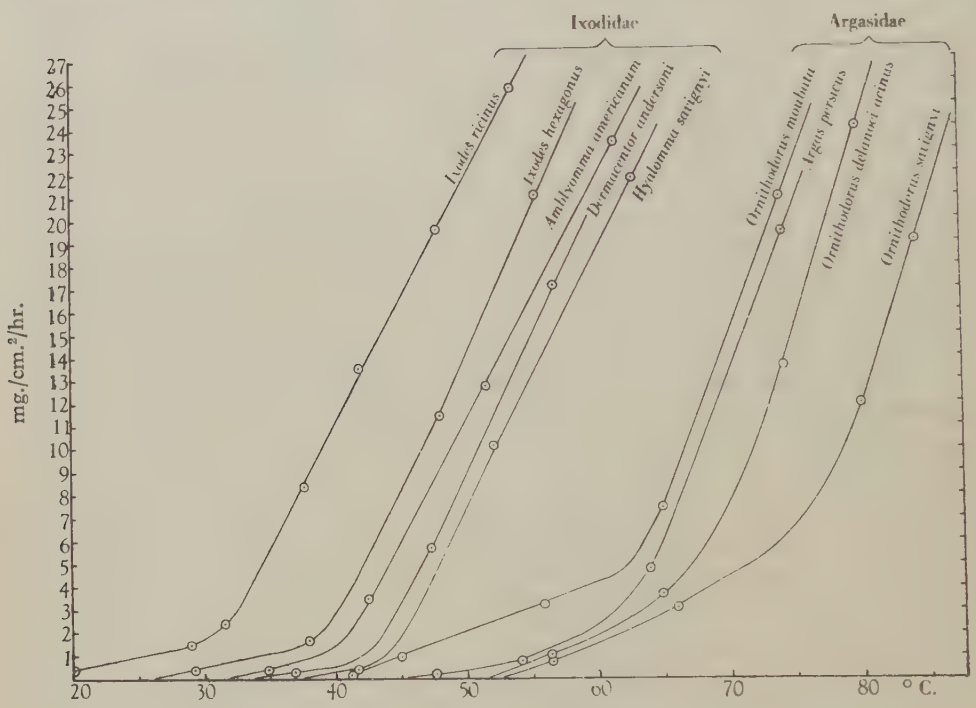

Fig. 1. The evaporation of water from dead engorged ticks at different temperatures.

Dermacentor, *Rhipicephalus* and *Hyalomma*. *Ixodes ricinus* emerges as an exception among the Ixodidae, having the low critical temperature of 32° C. In the argasid group there is little difference in the evaporation curves of *Argas persicus* and species of *Ornithodorus* such as *O. moubata*. *O. delanoei*, however, has a critical temperature higher by about 5° C., and *O. savignyi* by over 10° C.

A comparison of the results given in Table 1 where the ticks were desiccated for a longer period at a temperature well below the critical temperature, with the series given in Table 2 based on the evaporation curves, shows that the two arrangements correspond closely. As in the case of insects, therefore (Wigglesworth, 1945), those species most resistant to desiccation also have the higher critical temperatures.

The effect of temperature on transpiration through the cuticle of the unfed tick has been tested in *Dermacentor andersoni*. Fifteen unfed female ticks were killed in ammonia vapour, desiccated in the usual manner and weighed together. The resulting evaporation curve was almost identical with that of the engorged ticks of the same species. Clearly, therefore, a waterproofing layer with similar or identical properties is present in the unfed stage.

TRANSPIRATION AND ENVIRONMENT

Different species of ticks occupy a wide variety of 'ecological niches', and their powers of resisting desiccation are evidently adapted to meet the conditions normally encountered in their environment. All the species considered here leave the host after each blood meal* and are therefore exposed during most of their life cycle to the microclimatic conditions prevailing in the locality where they are dropped. The Argasidae, mainly nocturnal feeders, usually infest the host's burrow or nest. Ixodid ticks, which remain attached to the host for several days, are sometimes confined to similar haunts, but when the host is a wandering animal they tend to be dropped more or less at random over a wide range of territory. The type of vegetation and the microclimatic conditions it affords then becomes of considerable importance. There can be little doubt that comparatively small differences in transpiration will be of great significance from the point of view of distribution and ecology. The following notes illustrate the range of habitats occupied by some of the species under discussion: they may be considered in relation to the results summarized in Tables 1 and 2.

Owing to its susceptibility to desiccation, *Ixodes ricinus*, a common parasite of hill sheep in Britain, can survive only on rough hill and moorland grazings which provide a permanently moist microhabitat (MacLeod, 1936; Milne, 1944). The character of the vegetation varies, but in northern England the tick infests poorly drained grazings with rough grasses, bracken and rushes which, on dying back, yield a thick 'mat' of moist debris overlying the soil. The damp spongy cover afforded by heather associated with mosses also forms an ideal habitat. In Norway, *I. ricinus* is abundant only along the seaboard, on wet hillsides with deciduous woods and alder bushes or near the coast over swampy ground supporting junipers and heather (Tambs-Lyche, 1943).

Under natural conditions the European species *I. canisuga* is probably confined to the earths and burrows of mammals such as foxes and badgers and of birds like the sandmartin (Milne, 1947; Nuttall, Warburton, Cooper & Robinson, 1911). However, a particularly favourable, if secondary, host is the sheep dog, and the kennels of these animals are often heavily infested. In northern Britain the 'kennels' are often disused stables, and in a typical example in which meteorological records were kept, the humidity conditions were found to be much more adverse than are those

* With the exception of *Hyalomma savignyi* which is a two-host tick. In this species the engorged larvae moult on the host and the nymphs reattach immediately: only the larvae and adults seek a new host.

of the moist microclimate in which *I. ricinus* lives (unpublished observations). But *I. canisuga* has correspondingly greater powers of resistance.

Amblyomma americanum is also a relatively susceptible species. In Georgia and South Carolina where the chief hosts are deer and cattle, the tick occurs principally in the wooded coastal areas and particularly in the wetter parts of these areas.* Infestations are heaviest on small islands off the coast supporting woodland and salt marsh; and on the mainland they are heaviest near streams and in woodland which is lowlying and swampy. Ticks are more numerous in wooded areas with a dense stand of underbrush and a thick mat of decaying vegetation covering the soil than in localities where the brush is reduced and the mat absent.

Haemaphysalis punctata occurs in south-eastern England (Kent) as a parasite of sheep and other domestic stock. The tick is normally found in lush meadowland near the coast, but engorged ticks dropped by animals on temporary leys, which may have only a thin growth of grass affording little protection, can survive and develop for at least one season.

The three species of *Dermacentor* show somewhat greater powers of resistance. Their environment is similar in many ways. In localities of western England where the European species *D. reticulatus* is common, the tick is found infesting such places as the grassy verges of cliff tops, overgrown orchards or meadows with long permanent grass. The habitat of the American dog tick *D. variabilis* in Massachusetts is described by Smith, Cole & Gouck (1946). It occurs abundantly amongst beach grass along the shore, amongst roadside vegetation, and over rough ground with thick tangled grass and blackberry. Thick grass provides excellent cover for the meadow mice which are the hosts of the larval and nymphal stages. Typical situations for *D. andersoni* in the Bitter Root Valley of Montana are rocky scrub-covered slopes supporting a large rodent population (Cooley, 1932). In any of these localities the ground may become very parched during the summer months. The adults of *D. variabilis* are carried principally by dogs and are therefore sometimes dropped inside houses. Nevertheless, unlike *Rhipicephalus* (see below), this species does not survive in buildings unless high humidities are artificially maintained.*

The distribution of *R. sanguineus* in warm climates is cosmopolitan. Throughout the United States this species occurs principally in houses or other buildings where dogs are kept.* In the northern States the ticks survive in winter only if the buildings are kept heated. The tick can thus maintain itself in spite of the ensuing low humidities. In the southern States *Rhipicephalus* may infest yards as well as houses. In such cases the yards may be bare of grass or with merely a sparse covering of grass; yet in spite of exposure to the full drying effect of the sun the ticks survive readily.

The genus *Hyalomma* is predominantly African and Asiatic and is among the most resistant of the ixodid genera, several species extending into regions where very dry or even desert conditions prevail. *H. savignyi* is abundant in the settled regions of Palestine such as the Esdraelon Valley where cows are raised. In addition to infesting the live-stock enclosures in this district, some unfed ticks which have dropped

* Dr Carroll N. Smith (personal communication).

as the engorged stages from camels and other animals may be found running in the heat of the sun on sand dunes and over the surface of the desert near the Dead Sea.*

The Argasidae are much more richly represented in warm than in temperate climates, and even such species as extend into cooler regions choose dry, dusty habitats. The following are typical examples: the African species *Ornithodorus moubata* infesting the floors and walls of native dwellings; the North American species *O. parkeri* recovered from the nests of the burrowing owl, *Speotylo* (Jellison, 1940); *Argas persicus*, widely distributed in warm countries and a notorious pest of the poultry roost.

Finally, the exceedingly resistant Argasidae, such as *Ornithodorus savignyi* and *O. delanoei acinus*, exhibit an even greater degree of xerophily. *O. savignyi*, a species widely distributed in Africa, Arabia and elsewhere, is commonly encountered partly buried in loose dry sand near the resting places of caravans or in places where camels have been quartered. The very large tick, *O. delanoei acinus*, has been found in a similarly arid environment (Whittick, 1938).

ABRASION OF THE CUTICLE AND WATER LOSS

In insects impermeability is conferred by a film of wax or oil in the epicuticle, and if this layer is interrupted by rubbing the insect with an inert abrasive dust, such as alumina, water loss through the cuticle is enormously increased (Wigglesworth 1945). The same method has been applied to ticks with the object of demonstrating the waterproofing layer.

Table 3 shows the results obtained with three species. In each case the dorsum of the tick was abraded by drawing it several times over a length of filter-paper covered with alumina dust. It can be seen that this treatment greatly increases transpiration, both in unfed and engorged ticks.

Table 3. *The effect on water loss of rubbing the cuticle with alumina dust*

Species and treatment		Loss of wt. percent during 24 hr. in dry air at 25° C.
Ixodes ricinus:	Unfed female: control	17·0
	Unfed female, after abrasion	53·5
	Engorged female: normal	8·5
	The same after abrasion	54·0
Amblyomma cajennense:	Unfed female: normal	3·5
	The same after abrasion	50·6
	Engorged female: normal	2·0
	The same after abrasion	35·3
Ornithodorus moubata:	Engorged female: normal	0·6
	The same after abrasion	15·3

After abrasion with the dust the epicuticle appears undamaged, but in the engorged stages of some ticks (particularly in *Ixodes canisuga*) the cuticle is so smooth that the abraded areas appear as glassy patches from which the waxy bloom has been removed. These areas soon blacken as water evaporates and the gut, which is filled with darkened blood, begins to adhere to the epidermis.

* Dr B. Feldman-Muhsam (personal communication).

In most insects the presence of alumina in contact with the cuticle does not lead to increased transpiration unless there is actual abrasion; in the cockroach, however, merely sprinkling the dust on the body causes the mobile waterproofing oil to be adsorbed and thus increases water loss. Experiments in which ticks were suspended or otherwise prevented from moving showed that even if the dorsum was completely covered by dust there was no significant increase in evaporation. The following are examples of the results. In dry air at 25° C. an unfed female *Dermacentor andersoni* lost 1·4% of the original weight per diem before dusting and 1·3% after dusting. Comparable values for an engorged female *Ixodes ricinus* were 7·5 and 9·6%, and for *Ornithodorus moubata* 0·6 and 0·7%. This evidence therefore suggests that even in *Ixodes ricinus*, a species with a very low critical temperature, the waterproofing agent is a solid wax and not a mobile oil.

A similar increase in evaporation is observed if the dead tick is rubbed with dust. Since, however, water loss from the unfed tick increases after death, it is of interest to show that evaporation can be still further increased by abrasion. The following results have been obtained with unfed females of *Hyalomma savignyi*. Each value represents the average loss of weight from five unfed female ticks during 24 hr. in dry air at 25° C.

A. Alive	0·8
B. Killed in ammonia vapour	20·8
C. Killed in ammonia vapour and rubbed with alumina	57·2

After death evaporation increased from 0·8 to 20·8% of the original weight per diem; but the dead ticks abraded with alumina lost as much as 57% per diem.

Wigglesworth (1945) has shown that the extent of the abrasion of the wax layer in insects can be revealed by immersing the insect in ammoniacal silver nitrate, for reduction of the reagent then occurs only in those areas where the polyphenols in the epicuticle are exposed by the removal of the overlying wax layer. If a normal unfed tick is treated with 5% ammoniacal silver nitrate for 2 hr. no reduction of the reagent takes place except within the ducts of the dermal glands, which become filled with a conspicuous solid deposit of silver. Owing to the transparency of the larva it was possible to observe the formation of this curious precipitate under the microscope. The following observations were made on the larva of *Ixodes ricinus*.

About 1 min. after immersion in ammoniacal silver a minute granule appears in the centre of the lumen near the surface of the epidermal cell where it extends into the duct. The granule rapidly grows in diameter until after 15 min. it has formed a round plug entirely filling the duct. Eventually, as its growth continues, it may come to distend the duct and even push the epidermal cells away from the cuticle. Viewed by reflected light the precipitate at first appears as a white mass. Since a similar plug is also formed if the tick is immersed in a solution of silver nitrate in nitric acid, its formation may be due to the precipitation of chloride and not to the reduction of the silver by a polyphenol. Perhaps chloride is present in a thin film of moisture bathing the outer surface of the epidermal cell, and as it is precipitated by the reagent more chloride is withdrawn from the haemolymph.

Before describing the results of treating abraded ticks with ammoniacal silver it is

necessary to recall certain features of the cuticle structure. In ixodid ticks the integument shows considerable specialization in different regions of the body. Over the hard inextensible regions, such as the legs and scutum, the epicuticle is flat and

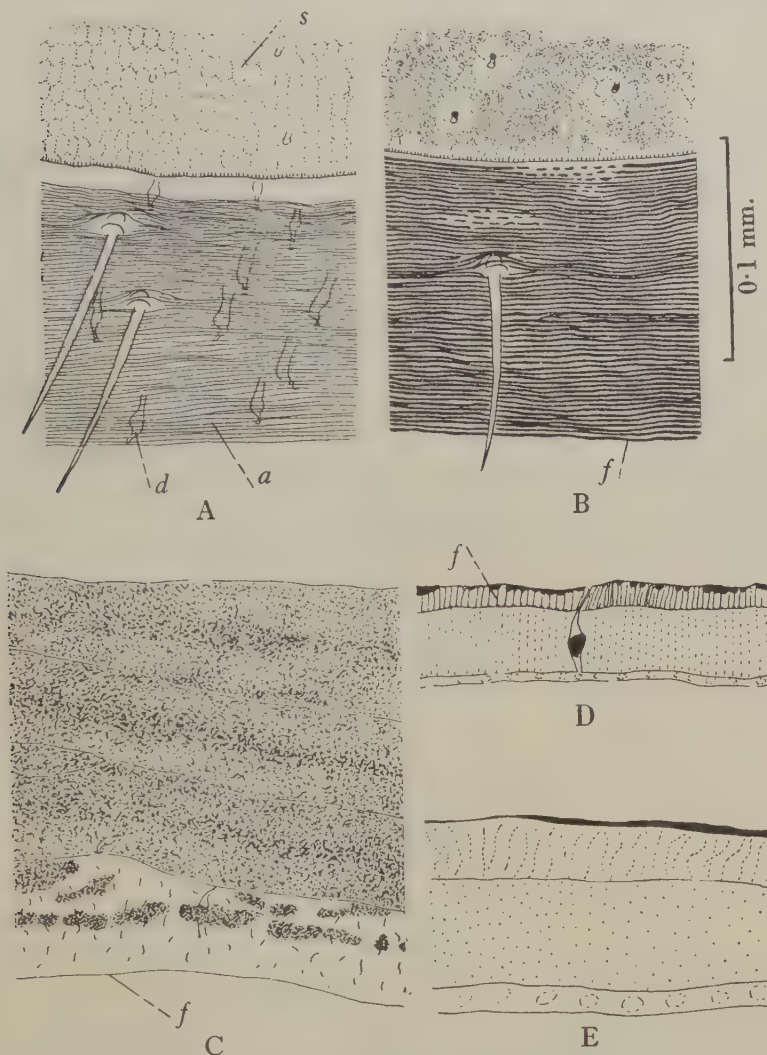

Fig. 2. The cuticle in *Ixodes ricinus*. A, surface view of the normal cuticle in the unfed female. B, the same after abrasion and exposure to ammoniacal silver. C, cuticle of the engorged female after the same treatment. D, E, sections of cuticle in unfed and engorged female ticks after treatment. *a*, alloscutum; *d*, duct of dermal gland; *f*, epicuticular folds; *s*, scutum.

the underlying 'endocuticle' dark and sclerotized down to the epidermis. Over the remaining parts of the body (the 'alloscutum') the epicuticle of the unfed tick is deeply folded to allow for expansion during feeding, while the endocuticle is unpigmented and elastic (Fig. 2A). The integument of Argasidae shows less regional

specialization and there is only one main type of cuticle. In *Ornithodorus moubata* the cuticle surface is raised into hemispherical tubercles, some of which are surmounted by bristles (Fig. 3A). Over the surface of the tubercles the epicuticle is smooth, but between adjacent tubercles it is raised into puckered folds which become flattened out after engorgement. The endocuticle is everywhere white and extensible. A further difference concerns the musculature. In Ixodidae the

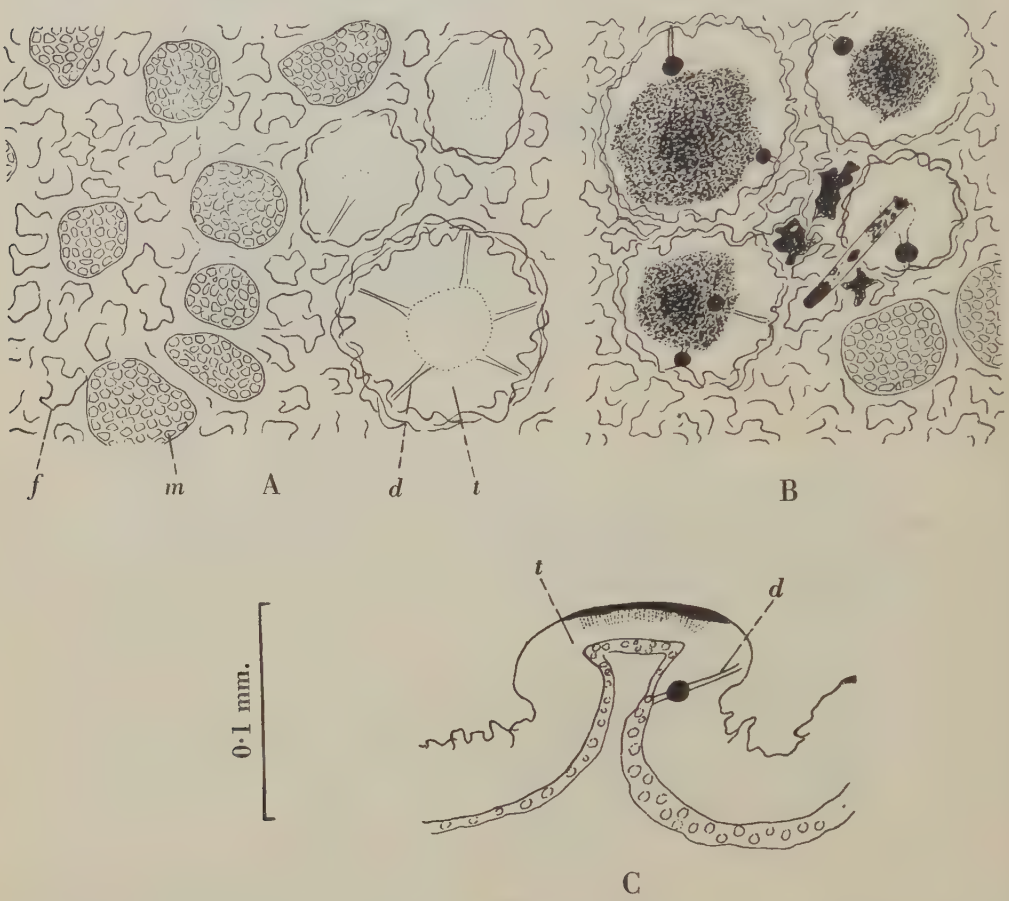

Fig. 3. The cuticle in *Ornithodorus moubata*. A, surface view of the normal cuticle. B, the same rubbed with alumina and exposed to ammoniacal silver. C, section of the treated cuticle. *d*, duct of dermal gland; *f*, epicuticular fold; *m*, muscle insertion; *t*, tubercle.

dorsoventral muscles are arranged in rows on the cuticle, but there is no modification in the pattern of the epicuticle overlying the insertions; externally their presence is marked only by the long furrows running over the integument. In Argasidae, on the other hand, the cuticle is considerably modified over the insertions. The boundary of each muscle bundle is clearly delineated and encloses a number of polygonal units representing the insertions of individual fibres (Fig. 3A).

After ticks have been rubbed in alumina dust and treated with ammoniacal silver, the epicuticle always shows considerable blackening over the abraded regions. The deepest staining occurs over the alloscutum of Ixodidae, even in those species, such as *Ixodes canisuga*, in which the epicuticle itself is colourless. There is less reduction over the hard dark areas. Clearly the epicuticle must contain polyphenols which in the normal tick are protected from reacting with the silver by the overlying waterproofing layer. In regions where the cuticle is heavily sclerotized there appears to be a corresponding reduction in free polyphenols. After abrasion the polyphenols are chiefly exposed over the elevated regions of the integument, particularly the crests of the epicuticular folds in unfed Ixodidae (Fig. 2B, D) and the surface of the tubercles in *Ornithodorus* (Fig. 3B, C). When the tick has fed and the epicuticle is smoothed out, reduction is much more widespread. In Ixodidae the whole epicuticle blackens over the abraded part (Fig. 2C, E); in *Ornithodorus* the dust is able to reach the folds between the tubercles. The affected bristles also show irregular patches of stain along their length.

Recovery after abrasion

When ticks are kept in damp air after being rubbed with alumina, they are able gradually to restore their impermeability. Some examples are given in Table 4. However, as Wigglesworth (1945) points out in the case of insects, the initial degree of impermeability is never fully recovered.

Table 4. *Water loss from living ticks after abrasion with alumina dust and recovery in saturated air*

	% loss of weight during 24 hr. in dry air at 25° C.
Dermacentor andersoni, unfed female, wt. = 5·6 mg.:	
Before abrasion	1·4
1 day after rubbing with alumina	18·6
4 days after rubbing with alumina	6·9
7 days after rubbing with alumina	5·0
Ornithodorus moubata, engorged female, wt. = 100·0 mg.:	
Before abrasion	1·2
1 day after rubbing with alumina	24·3
3 days after rubbing with alumina	4·8
5 days after rubbing with alumina	2·6
8 days after rubbing with alumina	2·6
	% loss of weight during 4 hr. in dry air at 25° C.
Ixodes ricinus, engorged female, wt. = 290 mg.:	
Before abrasion	0·9
1 day after rubbing with alumina	5·9
3 days after rubbing with alumina	3·0
5 days after rubbing with alumina	1·4
8 days after rubbing with alumina	1·5

The process of recovery, as revealed by immersing ticks in ammoniacal silver after different time intervals have been allowed for recovery in damp air, is similar in all species. In *Ornithodorus* the details can be followed on the smooth cuticle of

the tubercles. The whole of the abraded region darkens at first, but 20 hr. afterwards the staining area has retreated towards the summit of the tubercle, small outliers of stain remaining nearer the base. After 3 days staining is no longer perceptible, and the waterproofing layer again completely covers the underlying polyphenols.

The waxes of the epicuticle are secreted by the epidermal cells and are discharged by the pore canals. This can clearly be observed in engorged Ixodidae, such as *Ixodes ricinus*, where, during recovery from abrasion, the areas progressively covered bear no relation to the distribution of the dermal glands. The waterproofing layer cannot then be laid down by these glands, as has sometimes been held (Yalvaç, 1939).

The deposition of wax over the abraded regions is in other respects markedly dissimilar in the two families of ticks. In Ixodidae wax is deposited in an apparently

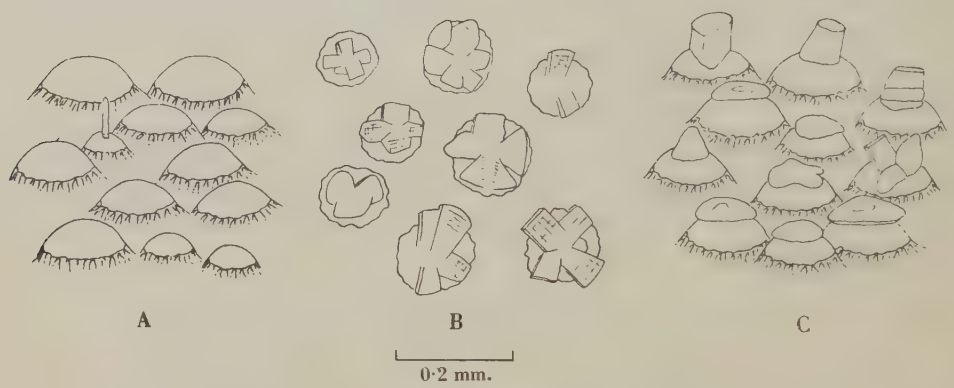

Fig. 4. The regeneration of wax in *Ornithodorus moubata* after rubbing the cuticle with alumina. A, normal cuticle viewed obliquely. B, cuticle seen from above (only tubercles shown) after recovery for 3 weeks in saturated air. C, oblique view of cuticle after abrasion and recovery at room humidity.

orderly fashion and after complete recovery has taken place in saturated air, deposition ceases. The cuticle then appears normal when viewed under the binocular. In *Ornithodorus moubata*, on the other hand, wax is laid down very irregularly and deposition apparently continues until moulting commences. In the adult (Fig. 4) enormous thicknesses of wax are secreted, chiefly over the tubercles which, as we have seen, are most liable to abrasion. These deposits of wax dissolve immediately in cold chloroform and are clearly unprotected by a further covering of cement (see below).

The humidity to which the recovering tick is exposed has a considerable influence on the growth habit of the wax deposits in *Ornithodorus*. Fig. 4B shows an example of recovery in saturated air. The wax filaments, as they have grown out from the surface of the epicuticle, have assumed a definite crystalline form, and many tubercles when viewed from the summit are seen to have a rosette of tabular wax crystals radiating from the summit. Often these crystals have striations running through them in the longitudinal and transverse planes. Deposits laid down in drier

air (recovery at room humidity) often lack any definite crystalline form (Fig. 4C) and usually appear as thick caps of wax with rounded contours perched on the summit of the tubercle. In 3 weeks the wax may have attained a thickness of nearly 100 μ, that is, greater than the entire thickness of the cuticle. There is no very conspicuous difference in the *quantity* of wax secreted in moist and dry air, such as there is in *Rhodnius* (Wigglesworth, 1945).

The effect of abrasion on the uptake of water through the cuticle

In a previous paper it was shown that after desiccation unfed ticks readily take up water through the cuticle when exposed to humidities near saturation (Lees, 1946). A covering of impermeable paint can be applied to any part of the body, including the spiracles, without preventing the uptake of water through the remaining uncovered cuticle. Presumably, therefore, the site of uptake is not localized in any way. Most unfed ticks reach a state of equilibrium at a humidity of about 90%. In view of these circumstances one would expect that if the wax layer were interrupted, water uptake would proceed normally from saturated air while at a humidity only slightly above the point of equilibrium, e.g. 95% R.H., uptake through the undamaged cuticle would be offset by the water lost to the unsaturated air through the abraded regions.

Unfed females of *Dermacentor andersoni* were used in investigating the effects of abrasion on water uptake. Now, if unfed ticks are desiccated repeatedly they take up water after each desiccation at very nearly the same rate when exposed to the same high humidity. Accordingly, the ticks were first desiccated individually in dry air at 25° C. for some 10 days and were then exposed in small containers to humidities of 95 or 100% R.H. The values for water uptake serve as the controls. The same ticks were then re-desiccated and abraded by rubbing the dorsum with alumina. After this treatment the dust was washed off in a stream of water, the ticks were carefully dried and reweighed and exposed again to the appropriate humidities. A sensitive torsion balance was used for weighing the ticks.

The results were unexpected (Fig. 5). After abrasion the desiccated tick fails to take up any water for 24 hr. or so, either from 95% R.H. or from saturated air. During this period uptake would normally be most rapid. The weight may sometimes fall slightly. After this initial delay the ability to secrete water is rapidly regained and uptake proceeds at the same rate as it did prior to abrasion. It is clear from this observation that the abrasion of a limited area of cuticle temporarily inhibits the activities of all, or nearly all, the epidermal cells, many of which underlie normal, undamaged cuticle. Evidently the epidermal cells are interconnected in some way, and the interruption in the function of one group of cells may influence the normal functioning of an adjacent group.

This effect of abrasion is not due to the fact that the epidermal cells are fully engaged in repairing the damaged waterproofing layer and are unable simultaneously to perform the task of secreting water. The full powers of water uptake are recovered long before the final degree of impermeability is restored, that is, before the deposition of wax over the abraded regions has ceased. The following

example will clarify this statement. An unfed female tick lost 1·3% of the original weight per diem in dry air at 25° C. and gained 8·8% during subsequent exposure to saturated air for 1 day. This tick was then re-desiccated, rubbed in alumina and exposed to saturated air. There was no increase in weight on the first day, but on the second day after the cuticle had been abraded the tick gained 9·8% in weight. During the third day the tick was exposed to dry air and lost 14·2% in weight; yet 15 days after rubbing with alumina the loss of weight per diem in dry air had fallen to 3·8%.

It is possible to establish an approximate relationship between the area of cuticle abraded and the suppression of water uptake. A single unfed female tick was

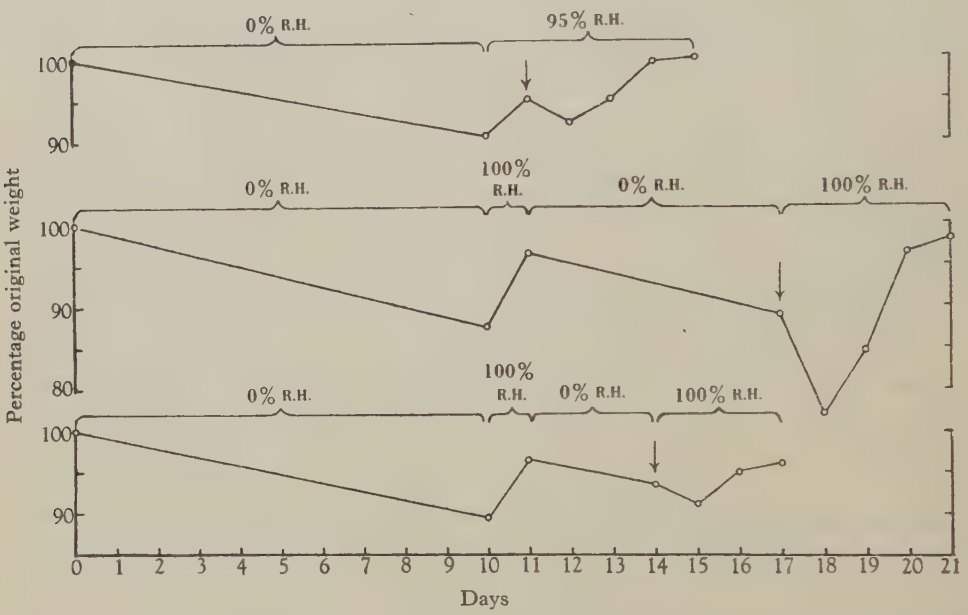

Fig. 5. The effect of abrasion on the uptake of water through the cuticle of *Dermacentor andersoni*. Results with three unfed female ticks are shown. The points at which the ticks were rubbed with alumina are marked by arrows.

desiccated and the rate of uptake in saturated air determined. After re-desiccation, an area of cuticle on the dorsum was marked out and thoroughly abraded by rubbing it with a tiny ball of filter-paper dipped in alumina. Uptake in saturated air was again determined. After several days had been allowed for recovery this procedure was repeated with a larger area of cuticle, and so on. The results showed that suppression does not become appreciable until a comparatively large area of cuticle has been abraded. In one example the abrasion of 6·1% of the total surface area only reduced uptake from 6·2 to 3·7% per diem; whereas when 15% was abraded uptake was reduced to 0·9%. This area was equal to nearly half the entire surface area of the dorsum and was underlain by an estimated 22% of the total epidermal cells.

The effect of chloroform and detergents on evaporation

Wigglesworth (1945) has shown that if whole insects are extracted with a wax solvent such as chloroform, evaporation through the cuticle is greatly increased. The precise effect in different insects depends on whether the wax layer is covered and protected from solution by an additional 'cement' layer, as in *Rhodnius*; also on the nature of the wax itself. In an insect with a soft, low melting-point wax transpiration is increased by mere exposure to chloroform vapour, but the latter has a much smaller effect in species waterproofed by hard, high melting-point waxes.

Similar experiments have been carried out with several species of ticks representative of the Ixodidae and Argasidae. Engorged females were killed in ammonia vapour and then either extracted with chloroform or exposed to chloroform vapour. Their spiracles were subsequently covered. The results are shown in Table 5. In

Table 5. *Percentage loss of weight from ticks during 4 hr. in dry air at 25° C. after chloroform extraction or exposure to chloroform vapour. The values for the controls represent percentage losses over 24 hr. periods*

Treatment	Ixodidae			Argasidae		
	Ixodes ricinus	*Dermacentor andersoni*	*Hyalomma savignyi*	*Argas persicus*	*Ornithodorus moubata*	*Ornithodorus savignyi*
Control	7·9	1·1	0·7	0·7	0·8	0·4
Exposed to chloroform vapour at 18° C. for 1 hr.	19·0	3·1	0·3	0·4	0·2	0·2
Extracted with chloroform at 18° C. for 1 hr.	26·8	14·8	12·9	7·3	0·7	1·9
Extracted with chloroform at 50° C. for 15 min.	32·0	22·1	16·0	46·2	35·8	19·0

Ixodidae with a low critical temperature, such as *Ixodes ricinus*, water loss is markedly increased by exposure to chloroform vapour alone. In *Dermacentor andersoni* and *Hyalomma savignyi*, both ixodids with much higher critical temperatures, chloroform vapour and even extraction in the cold has a much smaller effect. Argasid ticks, as typified by *Ornithodorus moubata*, are extremely resistant to chloroform vapour and to cold extraction; but water loss increases dramatically after extraction in chloroform at 50° C. The differences noted between *Ixodes* on the one hand and *Dermacentor* and *Hyalomma* on the other, are probably associated with the relative solubility of the waxes laid down by these species, for the wax layer does not appear to be covered by a further protective layer. In *Ornithodorus*, on the other hand, an insoluble layer of cement is present overlying the wax (see p. 396), and this protects the wax from solution except in hot chloroform when the cement is broken down.

The action of chloroform in attacking the waterproofing layer has also been studied by immersing ticks in ammoniacal silver after chloroform treatment. Engorged females of *Ixodes ricinus* show widespread staining over the surface of the epicuticle after chloroform extraction or exposure to chloroform vapour; in

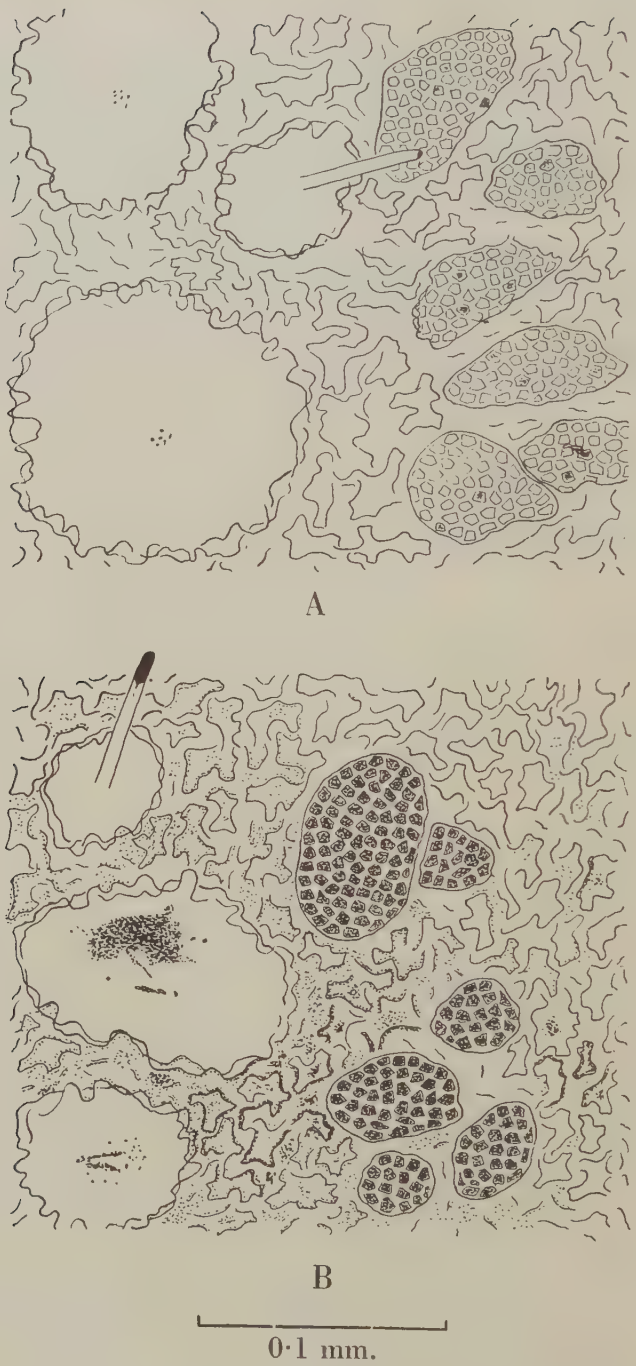

Fig. 6. Cuticle of *Ornithodorus moubata* after chloroform treatment and exposure to ammoniacal silver. A, after extraction in cold chloroform for 1 hr. B, after extraction in chloroform at 50° C. for 15 min.

Dermacentor the amount of staining after exposure to chloroform for a similar length of time is much less pronounced. If *Ornithodorus* is exposed to chloroform vapour for 1 hr. there is usually no visible reduction after silver treatment; and after extraction in chloroform at 18° C. for 1 hr. only a few very small black specks appear on the muscle insertions, at the tips of the bristles and on the tubercles (Fig. 6A). After 15 min. in hot chloroform, however, the muscle insertions blacken completely, the folds between the tubercles stain deeply along their crests and the bristles are often partly filled with a solid black deposit (Fig. 6B). There are usually patches of stain on the crowns of the tubercles, but in most specimens it is clear that by no means all the wax has been removed by this treatment. These observations support the conclusions drawn from the study of water loss after chloroform treatments.

It is known also that in insects transpiration can be increased if detergents are applied to the cuticle (Wigglesworth, 1945). Table 6 shows some results obtained with ticks. The full specifications of the materials used are given in the paper cited above; they were applied as a thin smear to the backs of living engorged female ticks.

Table 6. *Percentage loss of weight from engorged ticks smeared with various materials and exposed to dry air at* 25° C. *for* 24 *hr.*

Material	*Ixodes ricinus*	*Amblyomma cajennense*	*Ornithodorus moubata*
Control	7·9	1·4	0·8
P31	41·9	2·0	0·3
Diglycol oleate	45·0	2·6	2·2
R2211	49·4	4·5	3·8
C09993	53·2	12·3	4·2

P31, a refined mineral oil, greatly increases evaporation in *Ixodes ricinus* but has little or no effect in more impermeable species such as *Amblyomma cajennense* and *Ornithodorus moubata*. The wax detergents diglycol oleate and the cetyl ethers of polyethylene glycol, R2211 and C09993, show progressively greater powers of increasing transpiration, as they do in insects. But none of these materials is outstandingly effective in *Amblyomma* and they are almost without effect in *Ornithodorus*.

THE STRUCTURE OF THE EPICUTICLE

The nymphal exuvium of ticks consists almost entirely of the old epicuticle which, apart from the hard cuticle of the legs and scutum in Ixodidae, alone remains undigested by the moulting fluid. The exuvia of *Ixodes ricinus* and *Ornithodorus moubata* show many of the chemical characteristics of typical insect epicuticles—for example, insolubility in cold mineral acids and solubility in hot caustic potash. If nymphal skins of either species are extracted for 1 hr. in boiling chloroform and are then warmed gently in nitric acid and potassium chlorate, there is at first some evolution of gas from the inner side of the cuticle. On further warming the membrane expands and begins to disintegrate, liberating oily droplets which are themselves readily oxidized. Evidently a fatty material which cannot be extracted with lipoid solvents is incorporated in the epicuticle. The results of this test are

similar to those described by Wigglesworth (1947) in *Rhodnius*, and it is likely that the rigid basal layer of the epicuticle in ticks is similar to, if not identical with, the 'cuticulin' layer of insects. This substance is provisionally regarded by Wigglesworth as a polymerized lipo-protein subsequently tanned by quinones.

Treatment with ammoniacal silver after abrasion, or chloroform treatment, shows that in all ticks the cuticulin layer is covered by a film of polyphenols which in turn is overlain by the waterproofing layer. In *Ornithodorus moubata* the wax layer is covered by a further layer of cement which, as we have seen, resists extraction in cold chloroform. This layer can be demonstrated readily in paraffin sections which have been cleared in xylol. Fig. 7 shows the cuticle in a moulting tick; the

Fig. 7. Section of the cuticle of a moulting last stage nymph of *Ornithodorus moubata*. Cleared in xylol, the section shows the cement layer fraying away from the surface of the old cuticle. Note the absence of cement over the new cuticle. *c*, cement layer; *e*, epicuticle (cuticulin layer); *ex*, exuvial cavity; *ic*, imaginal cuticle; *nc*, nymphal cuticle.

wax layer has been dissolved from the old cuticle and the thin covering of cement (*c*) has frayed away from the surface. It will be noticed from this figure that a similar layer is absent from the new cuticle although moulting is far advanced. The cement layer itself forms a perfect mould of the contours of the epicuticle apart from its seeming absence over the bristles. Seen in section the cement appears to be of very uniform thickness, but the fact that hot chloroform exposes the polyphenols with much greater ease over the muscle insertions than over the puckered folds and the tubercles, suggests that this layer is actually thinnest over the muscle insertions.

No evidence of the presence of a cement layer was obtained in any of the ixodid ticks examined. Sections of the cuticle of *Ixodes ricinus*, *I. canisuga* and *Hyalomma savignyi* were prepared after clearing in xylol, but no detached superficial layer could be detected. The conclusion that the wax layer in Ixodidae is unprotected by a further cement covering, is, as we have seen, supported by the results of the chloroform extractions.

THE DEPOSITION OF THE WATERPROOFING AND CEMENT LAYERS

With the object of determining the time relations and mode of deposition of the waterproofing layer, moulting nymphal ticks were dissected out of their old cuticles and immersed in ammoniacal silver. In *Rhodnius* a picture of the extent of wax deposition can be derived from this technique, as the polyphenols are secreted before the overlying waterproofing layer (Wigglesworth, 1947). Three species of ticks were examined, namely, *Ixodes ricinus*, *Dermacentor andersoni* and *Ornithodorus moubata*. Since the duration of the moult at constant temperatures often showed considerable variations, it has been convenient to distinguish four stages as a broad guide to the general course of development. These are as follows:

A. From the beginning of engorgement to the time of separation of the epidermis from the old cuticle.

B. From the end of A to the time of onset of the secretion of moulting fluid. During this period the cuticulin layer is laid down.

C. From the end of B to the time of withdrawal of the moulting fluid.

D. From the end of C to emergence.

During stage B, that is, before the appearance of moulting fluid, it is almost impossible to carry out the dissection without damaging the new cuticle. If pieces of cuticle at the end of stage B are exposed to ammoniacal silver without previous fixation some ill-defined staining may develop in the epicuticle; but in many preparations no blackening occurs and polyphenols may be absent. Shortly afterwards, as moulting fluid begins to appear, the old cuticle can be removed successfully and the new cuticle exposed to silver from the outside. In the three species investigated there is comparatively little staining even at this early stage. But if the cuticle is first rubbed with alumina there is widespread reduction over the elevated regions. This shows that not only is the polyphenol layer fully established, but a large part of it is already covered by the waterproofing layer. Thus in ticks the waterproofing layer is laid down comparatively early in development, probably immediately after the secretion of the moulting fluid.

The waterproofing layer is never quite complete, however, at the beginning of stage C. In *Ornithodorus* the muscle insertions and some of the stellate folds blacken completely, and there is some reduction over the summits of the tubercles and at the bristle tips (the silver stain often appears in the form of spiky crystals) (Fig. 8A). After the withdrawal of the moulting fluid the extent of the silver staining gradually diminishes, the last region to be covered by the waterproofing layer being the muscle insertions. This takes place just before emergence (Fig. 8B). In *Ixodes* and *Dermacentor* the deposition of wax is even further advanced at the stage when moulting fluid is abundant. Exposure to silver results in some irregularly distributed patches of stain appearing over the scutum and alloscutum; but their total area is extremely small when compared with the area of cuticle over which the polyphenols are protected from staining (Fig. 9). After the moulting fluid has disappeared these areas are soon covered over.

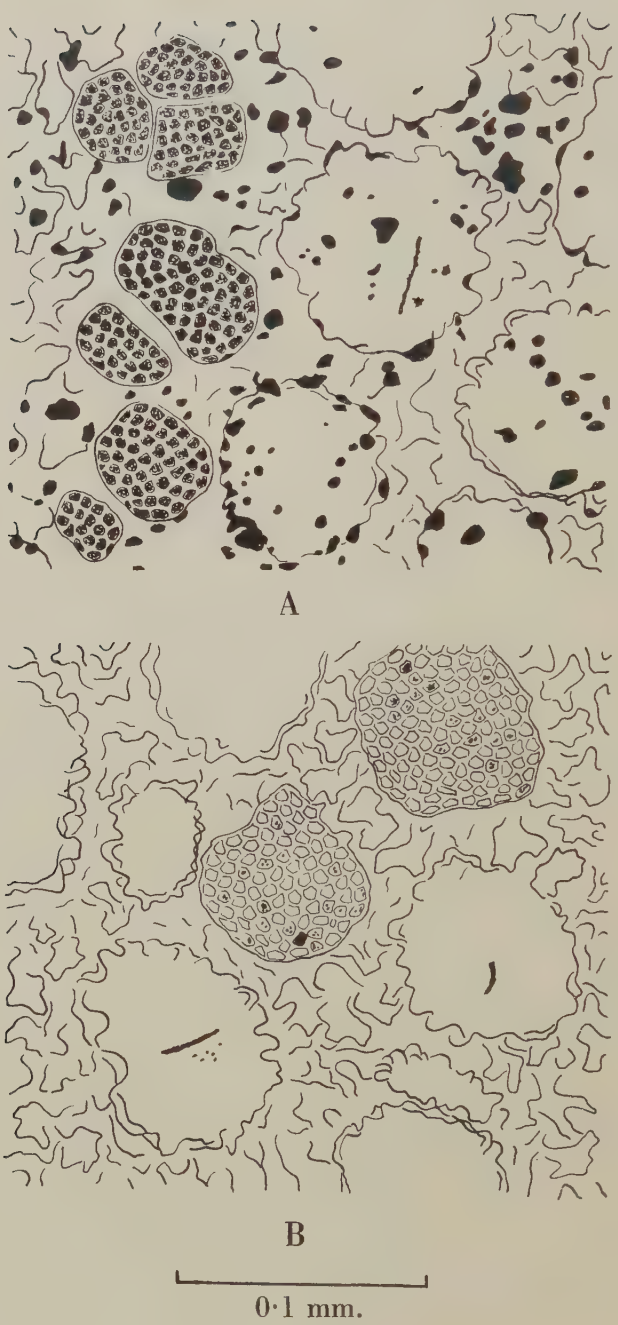

Fig. 8. The extent of the deposition of the wax layer in moulting last stage nymphs of *Ornithodorus moubata*. The old cuticles were dissected off and the ticks immersed in ammoniacal silver. A, stage C, abundant moulting fluid present. B, stage D, a few hours before emergence; moulting fluid withdrawn and cuticle hydrophobic.

The waterproofing layer is deposited at such an early stage that some doubt arises as to how the moulting fluid is withdrawn. Possible sites of absorption are the external openings, including the mouth and spiracles, the whole cuticle, or the small patches of cuticle over which the waterproofing layer is still incomplete. An attempt to determine the site of withdrawal in *D. andersoni* by injecting neutral red into the ecdysial fluid of moulting nymphs was only partly successful; for although the dye soon appeared in the Malpighian tubes as the fluid was withdrawn, no concentrations of dye were visible in the cuticle or elsewhere. On the whole it seems more

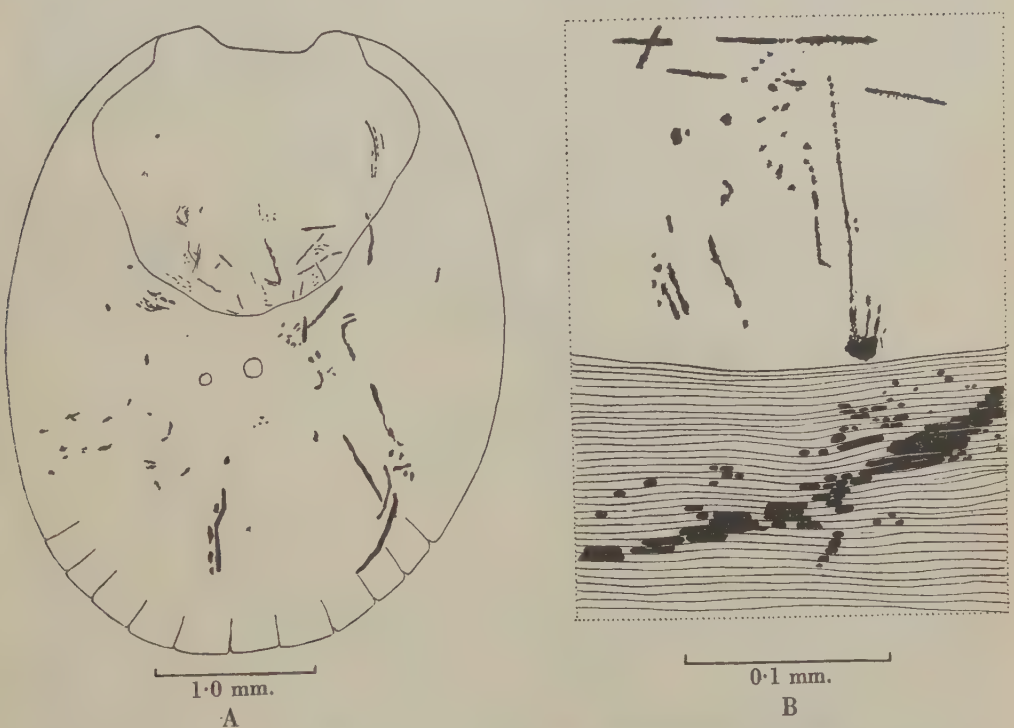

Fig. 9. The extent of the deposition of the wax layer in a moulting nymph of *Dermacentor andersoni* during stage C. Moulting fluid was abundant. After dissecting off the old cuticle the tick was immersed in ammoniacal silver. A, the entire dorsum, showing the extent of the reduction by polyphenols. B, detail of the staining over a smaller area.

likely that the fluid is withdrawn through those parts of the cuticle which happen to remain unwaterproofed.

The hydrophobic properties of the cuticle in *Ixodes* and *Dermacentor* are such as would be expected from the foregoing remarks. If moulting nymphs are dissected out of the nymphal exuvia and the moulting fluid blotted from the surface of the imaginal cuticle, droplets of water introduced on to the surface by means of a fine pipette fail to wet it. And after moulting the cuticle remains strongly hydrophobic. In *Ornithodorus* the properties of the cuticle are more complex. In the presence of moulting fluid the cuticle is easily wetted by water, and after its withdrawal and

replacement by air it remains hydrophilic for a time and then becomes highly hydrophobic. After emergence the cuticle remains hydrophobic unless the tick is immersed in water, a treatment which again renders the cuticle hydrophilic. This may normally occur when the tick feeds and becomes bathed in its own coxal fluid. The lessening affinity of the cuticle for water after the withdrawal of the moulting fluid is not associated with the increasing extent of wax deposition, for treatment with silver often showed that deposition was further advanced in cuticles that were easily wetted than in others which had already become hydrophobic. Presumably the waterproofing agent in this species contains mobile polar as well as apolar elements. In the presence of the moulting fluid the hydrophilic endings in the surface will be oriented outwards, but as air replaces moulting fluid the outwardly directed endings become predominantly apolar. The increased affinity of the cuticle for water following immersion is more difficult to account for, however, as by this time the wax layer has acquired a further covering of cement.

Table 7. *Percentage loss of weight during 4 hr. in dry air at 25° C. of last instar nymphs of* Ornithodorus moubata *at different developmental stages. A, untreated; B, after extraction in chloroform at 18° C. for 1 hr. Ticks which had not yet emerged were dissected out of the old cuticles*

Stage of development	A	B
Moulting fluid present	25·7	39·8
Moulting fluid withdrawn; cuticle hydrophilic	4·7	21·7
Moulting fluid withdrawn; cuticle hydrophobic	2·2	23·3
30 min. after emergence	1·8	17·6
15 hr. after emergence	0·7	9·3
3 days after emergence	0·7	1·6

The cement layer in *Ornithodorus* is laid down shortly after moulting. This can be shown by extracting ticks in different stages of development with cold chloroform and by estimating water loss through the cuticle. These results have been compared with the water loss from unextracted ticks in the same stage of development (Table 7). Transpiration through the new cuticle is always rapid during stage C when moulting fluid is present. After its withdrawal and the completion of the waterproofing layer impermeability becomes fully established; but until the time of emergence transpiration is greatly increased by cold chloroform extraction. Within a few hours of moulting, however, the effect of extraction begins to diminish, indicating that the cement is being laid down. In ticks which have moulted 1 hr. previously, extraction with chloroform exposes the polyphenols very easily over the muscle insertions and to a varying extent along the folds and summits of the tubercles. 15 hr. after moulting the waterproofing layer is usually completely protected from extraction. The time of deposition of the cement layer is therefore much the same as in *Rhodnius* (Wigglesworth, 1947).

THE DERMAL GLANDS

At certain stages in the developmental cycle of ixodid ticks the dermal glands discharge a visible product on to the surface of the cuticle. The material in question is a thick yellow 'grease' which slowly spreads over the cuticle or collects round the gland openings (Fig. 10). It is never discharged by the unfed tick and only begins to appear a few days after full engorgement. But even in the same species some stages may fail to discharge the grease. None of the material is visible in recently engorged

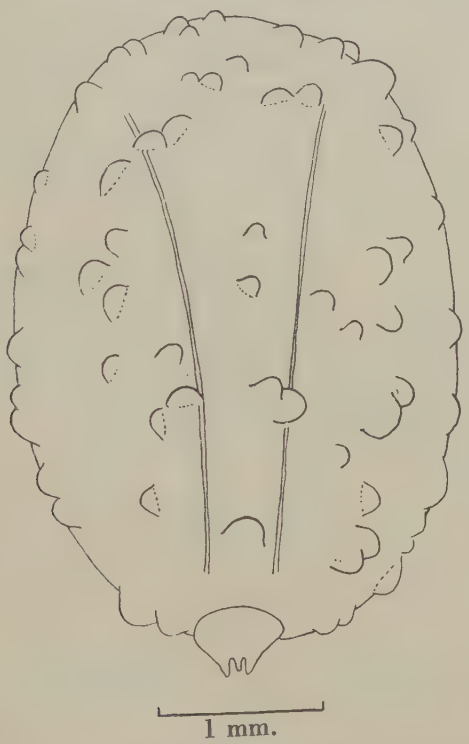

Fig. 10. Engorged nymph of *Dermacentor andersoni* about 1 week after dropping from the host. The tick has been kept in moist air and shows large masses of yellow 'grease' and water collected round the openings of the dermal glands. This material has no waterproofing function and is derived from the involuted dermal glands.

larvae and nymphs of *Ixodes ricinus*, for example, while there is a copious discharge from the dermal glands of the engorged, egg-laying female. In *Dermacentor andersoni* and other species, on the other hand, there is an abundant greasy exudate from the glands of the engorged nymph (Fig. 10). In Argasidae no products visible to the unaided eye are ever secreted by the dermal glands.*

It has been suggested that the function of the greasy material in *Ixodes ricinus* is to

* Schulze's (1942) assertion that the dermal glands are absent in the Argasidae is incorrect.

waterproof the cuticle (Falke, 1931) and, similarly, in *Hyalomma anatolicum* to protect the cuticle and 'sense organs' from desiccation (Yalvaç, 1939).*

In *Ixodes ricinus* the yellow grease is easily removed from the cuticle of the egg-laying female by wiping the surface with extracted cotton-wool. The material proved to be completely insoluble in cold chloroform and xylol, and only sparingly soluble in hot chloroform. Since it is known that all insect waterproofing agents are readily soluble in chloroform (Beament, 1945), and that in *Ixodes* also chloroform extraction removes the waterproofing layer from the cuticle (p. 393), it is most unlikely that the dermal gland exudate has a waterproofing function. Indeed, as has already been indicated, the true waterproofing layer is laid down by the epidermal cells and is completed before moulting. The histological development of the dermal glands in *Ixodes* and *Dermacentor* has been followed with the object of throwing light on the significance of the greasy exudate. In *Ornithodorus* the histology of the glands has been examined in relation to their probable function in secreting the cement layer. The results are described below.

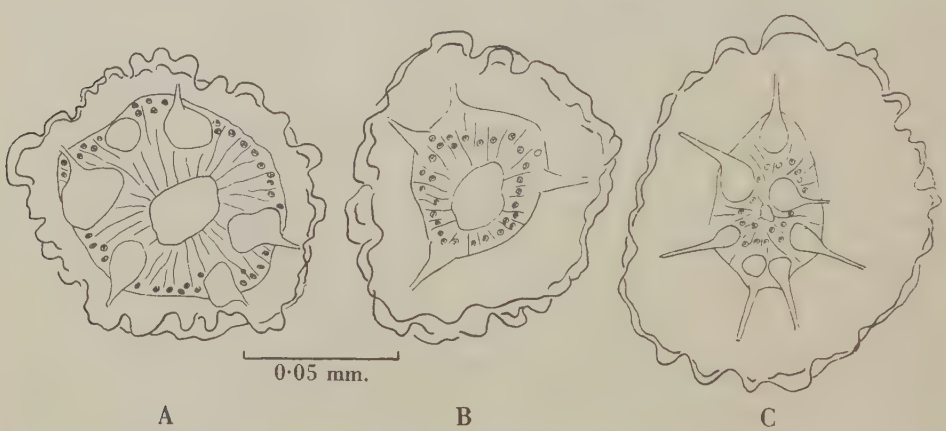

Fig. 11. Single tubercles in moulting nymphs of *Ornithodorus moubata* showing the appearance of the dermal glands at different developmental stages. A, shortly before the appearance of the moulting fluid. B, after withdrawal of the moulting fluid. C, female, 2 hr. after emergence.

Ornithodorus moubata

The openings of the dermal glands lie round the periphery of the tubercles, and the ducts, numbering from one to eight according to the size of the tubercle, run inwards to the epidermis which forms a deep pocket extending into the interior of the tubercle (Fig. 3C). The dermal glands can best be examined in whole preparations of the cuticle by focusing downwards through the thickness of the tubercle. At most stages during the development of the last nymphal instar the dermal glands remain invisible. Signs of activity become apparent, however, at two stages in the moulting cycle: just before the secretion of the moulting fluid (Fig. 11A) and

* Yalvaç (1939) and Schulze (1942) regard the dermal glands in Ixodidae as sense organs with an additional glandular function ('drüsensinnesorgane').

a few hours after moulting, as the cement layer is being deposited (Fig. 11C). The histological appearance of the glands is very similar during both phases of activity, the lumen gradually filling with a colourless secretion. During the latter phase of activity the glands appear never to attain the distended and conspicuous condition of the dermal glands in the fourth stage nymph of *Rhodnius* shortly before the deposition of the cement layer (Wigglesworth, 1947).

Dermacentor andersoni

The dermal glands in this species undergo a complicated cycle of activity, the significance of which is by no means clear. There are two types of gland each provided with an elaborate cuticular duct of characteristic form. From the description by Yalvaç (1939) of the dermal glands ('drüsensinnesorgane') in *Hyalomma anatolicum* it is clear that the larger type with a wide neck and a frilled opening corresponds with his 'sensillum sagittiforme', and the smaller, which has a narrow neck and a slit-like opening, with his 'sensillum hastiforme'. In common with other species of Ixodidae the gland cells in *Dermacentor* attain a gigantic size at certain stages in the developmental cycle. The general fate of the glands has been followed through from the nymphal to the adult stages.

The large dermal glands (type A) may be considered first (Fig. 12A–D). In the unfed nymph two gland cell nuclei, somewhat larger than the other epidermal nuclei, are associated with each duct; but the cytoplasm is either very sparse or fails to stain with haematoxylin (Fig. 12A). As soon as the tick begins to feed, however, the gland cell nuclei hypertrophy and secrete abundant deeply staining cytoplasm (Fig. 12B). By the end of engorgement the glands have attained their maximum size. There are usually two gigantic cells associated with each duct, but sometimes there are three such cells, sometimes only one (Fig. 12C). The cytoplasm of the cells is often somewhat fibrous, and the nucleus, which may be flattened, often rests against a large central vacuole containing fluid. The fate of the cells depends on the general course of development. If the tick fails to moult, the gland cells may persist unchanged for a long period; but if the moult is impending the cells soon begin to show degenerative changes. The cytoplasm becomes yellowish and reticulated and yellow greasy droplets appear (Fig. 12D). The gland then undergoes involution, the yellow residue passing up the duct to the surface of the cuticle. This is the greasy material already referred to and as such it clearly represents nothing more than the end-product of the degenerating gland cells. Sometimes the gland-cell nuclei become pycnotic and are thrown out with the cytoplasm; more frequently they remain resting against the epidermis and only the cytoplasm is lost. After the epidermis has separated from the old cuticle a group of small cells near the neck of the old gland are associated in secreting a new duct. A few of the old dermal gland nuclei may still persist beneath the epidermis when the tick moults.

The cells of the small dermal glands (type B) undergo a somewhat different cycle of activity (Fig. 12E–I). In the unfed nymph their component nuclei are indistinguishable from the other epidermal nuclei and only the cuticular duct can be recognized at this time (Fig. 12E). During engorgement, nuclei near each duct

hypertrophy and begin to accumulate cytoplasm, just as in the case of type A cells. Again also, maximum size of the paired cells is attained at the end of engorgement (Fig. 12F). After the tick has dropped from the host the type B cells undergo very rapid involution and a few days afterwards, when many type A cells are still

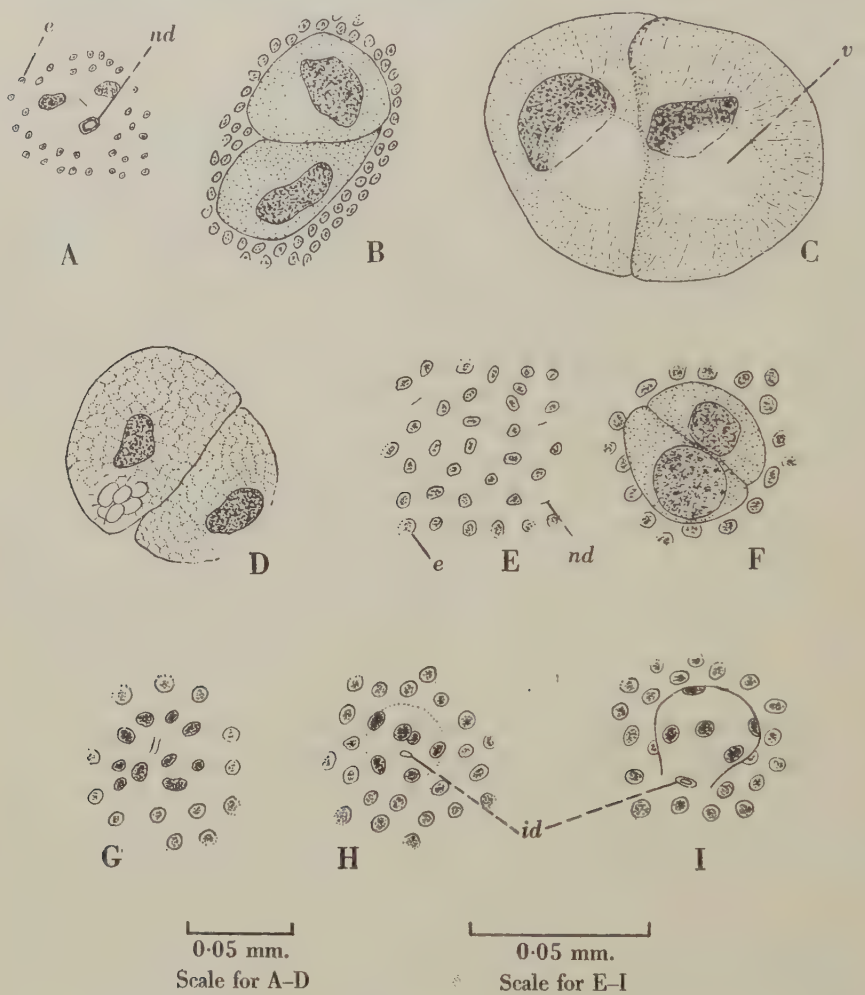

Fig. 12. Stages in the development of the dermal glands in *Dermacentor andersoni*. Surface views of the epidermis in the developing nymph. A–D, development of the large dermal glands (type A). E–I, development of the small glands (types B and C). A, unfed nymph. B, partially engorged nymph. C, engorged nymph, 1 day after dropping: maximum development of the paired gland cells. D, engorged nymph, 1 week after dropping: paired type A cells undergoing involution. The epidermis had not yet separated from the old cuticle in this tick. E, unfed nymph. F, engorged nymph, 1 day after dropping: maximum development of paired type B cells. G, engorged nymph, 1 week after dropping. The paired type B cells have degenerated and disappeared but moulting has not yet begun. H, I, development of a multinucleate type C gland cell from a group of nuclei near the future duct: the epidermis has now separated from the old cuticle and the cuticulin layer of the adult is being laid down. *e*, nuclei of epidermal cells; *id*, imaginal duct of dermal gland; *nd*, nymphal duct of gland; *v*, vacuole.

persisting unchanged, all the type B cells have disappeared, the products of the degenerating glands again passing up the ducts to the surface of the cuticle. There remains near the old duct a group of about eight nuclei which stain more deeply with haematoxylin than the other epidermal nuclei (Fig. 12G). Soon afterwards the moulting tick frees the epidermis from the old cuticle. The nuclei remain grouped round the new cuticular duct as it is forming and begin subsequently to secrete cytoplasm. Each group of nuclei thus participates in the formation of one new type of gland cell (type C). These are round or pear-shaped and their cytoplasm is acidophil and without inclusions. The multinucleate type C cells attain a maximum diameter of about 30μ shortly before the moulting fluid is secreted (Fig. 12I). Afterwards they dwindle in size but the groups of nuclei are still visible when the tick moults.

Ixodes ricinus

Only one type of dermal gland is present and this resembles the small type B gland of *Dermacentor*. The general course of development is also similar in many ways (Fig. 13). As the nymph becomes engorged the gland-cell nuclei, hitherto indistinguishable from the other epidermal cells, secrete a large quantity of basophilic cytoplasm. The paired cells again reach their maximum size at the end of engorgement when large vacuoles begin to appear near the nuclei. The neck cell nuclei are clearly seen at this time grouped round the duct (Fig. 13B). As in *Dermacentor* the appearance of vacuoles seems to be a sign of impending degeneration, but the cells persist entire until moulting has begun and the epidermis separated from the old cuticle. The cells then undergo involution (Fig. 13C). Since, however, the glands are now unprovided with ducts the products of degeneration cannot escape to the surface of the old cuticle, but are absorbed instead. Because of this difference in timing the degenerative products of the nymphal gland cells never become visible externally.

The cytoplasm of the type B cells disappears before the nuclei, which may persist until the cuticulin layer has been secreted (Fig. 13D). The neck cell nuclei remain near, and possibly secrete, the new cuticular duct. They appear to accumulate a little clear cytoplasm but undoubted multinucleate gland cells, like the type C cells of *Dermacentor*, have not been distinguished with certainty. After the secretion of the moulting fluid the epidermal nuclei become smaller, and when the tick emerges the epidermis again consists of a uniform sheet of cells; all the nuclei are of the same size and no cell boundaries are visible.

When the female tick becomes engorged the cycle of hypertrophy and involution is once more repeated (Fig. 13E–H). Since the adult does not moult, however, the products of the degenerating gland cells are not absorbed but are thrown out on to the surface of the cuticle, appearing as the characteristic yellow greasy material.

The development of the type A gland cells in *Dermacentor* is probably very similar to that of the similar cells in *Hyalomma* (Yalvaç, 1939); this author describes type A cells in engorged ticks discharging their 'secretion' on to the surface of the cuticle, the separation of the epidermis from the old cuticle and the subsequent

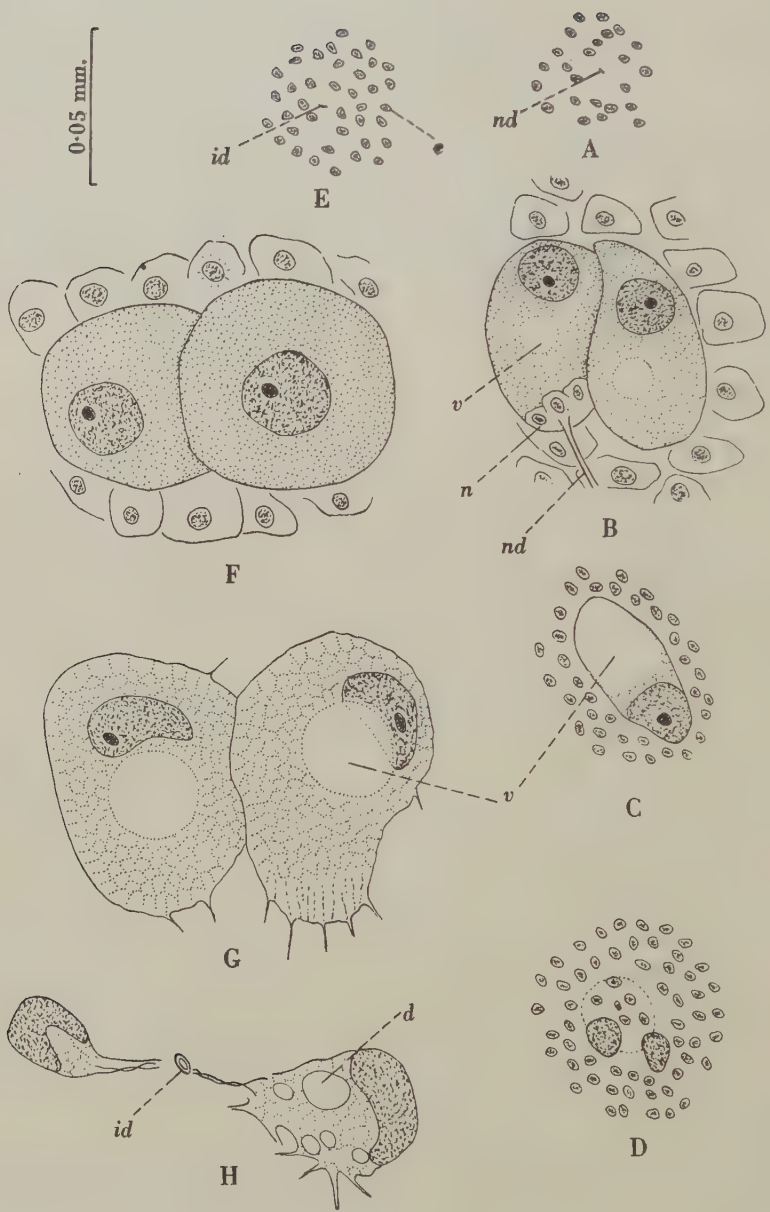

Fig. 13. Stages in the development of the dermal glands in *Ixodes ricinus*. Whole preparations of the epidermis. A, unfed nymph: the future gland cell nuclei cannot be distinguished. B, engorged nymph one day after dropping: maximum size of paired gland cells in the nymph. C, a degenerating gland cell in a moulting nymph: the epidermis had separated from the old cuticle. D, moulting nymph showing two persistent gland cell nuclei. E, unfed female. F, engorged female one day after dropping. G, H, degenerating dermal glands in a female tick, shortly after the onset of oviposition. *d*, droplets in the degenerating cell; *e*, nuclei of epidermal cells; *id*, imaginal duct of dermal gland; *n*, neck cells; *nd*, nymphal duct of dermal gland; *v*, vacuole.

'regeneration' of the gland cells. The regenerated glands figured (Yalvaç, 1939, figs. 30–32) are typical type A glands each consisting of a pair of gigantic vacuolated cells. Type C gland cells were not described. Yalvaç holds the view that the dermal glands have two main functions, namely, the secretion of a waterproofing material and the elaboration of moulting fluid by the 'regenerated' gland. Now their appearance is so similar in *Dermacentor* that one may question whether the glands described by Yalvaç in *Hyalomma* were not all type A cells in different stages of degeneration. As mentioned above, a few of these glands may persist beneath the epidermis after it has separated from the old cuticle. Schulze (1942) regards the cuticular ducts of the dermal glands as sense organs. It should be mentioned that his 'sense cells', as figured for *Ixodes ricinus* (Schulze, 1942, Fig. 7) are identical with the neck cells which remain after the type B cells have degenerated (Fig. 13 B). Their sensory function has never been demonstrated.

The true functions of the dermal glands in Ixodidae are therefore in doubt. Clearly the glands of types A and B do not secrete the waterproofing layer for these glands only develop as the tick becomes engorged, whereas the true waterproofing layer is completed before moulting. Neither do they secrete the moulting fluid for nearly all of them undergo involution before the epidermis has separated from the old cuticle and long before any moulting fluid appears. The period of hypertrophy as the tick feeds coincides with a growth phase undergone by all the epidermal cells which at this time are concerned with the renewed synthesis of cuticular material. However, it is uncertain whether A and B cells actually secrete the inner part of the duct which is continued through the thickening cuticle, or whether this, like the outer, amber-coloured part of the duct, is laid down by the group of small neck cells. Type C cells may secrete, or participate in secreting, the moulting fluid. It is noteworthy that degeneration of the dermal glands also occurs in the adult *Rhodnius* where the products of involution form little patches of 'secretion' near the openings of the ducts.

DISCUSSION

The greatly increased transpiration which follows abrasion of the cuticle shows that ticks owe their powers of resisting desiccation primarily to a superficial waterproofing layer. The visible products secreted during recovery from abrasion leave little doubt that the waterproofing agents are in all cases true waxes, even in the less resistant species, such as *Ixodes ricinus*. The waterproofing system therefore closely resembles that of the majority of insects (Wigglesworth, 1945).

The waterproofing layer in ticks forms a physical barrier to evaporation and as such is an essential feature in their water economy. But in unfed ticks the passive retention of water is assisted by active secretion. This faculty depends on the normal functioning of the epidermal cells and is displayed when the tick is exposed to any humidity below saturation; under these conditions the tick can resist water loss more effectively than it can by virtue of the wax layer alone—as is shown, for example, by increased transpiration if the tick is killed (Lees, 1946). Although the active and passive mechanisms operate towards the same ends in retaining water, it is

worth noting that if the waterproofing layer in the living tick is interrupted by abrasion, secretion by itself is incapable of preventing rapid water loss.

If the humidity to which the living tick is exposed is higher than the 'equilibrium humidity', secretory activity results in water being taken up through the cuticle (Lees, 1946). As the previous work had indicated, the waterproofing layer, although effectively preventing water loss does not hinder the uptake of water in the reverse direction. Indeed after injury to the cuticle by abrasion, water uptake by the whole epidermis is entirely upset for a time—a phenomenon which may perhaps be likened to wound shock. And after recovery of the full powers of secretion, water uptake proceeds at the same rate irrespective of whether the wax layer is still incomplete or whether it has been fully repaired. The mechanism of uptake is unknown. Even if some part of the epicuticle serves as a condensing system, water withdrawn from moist air will still have to pass through the wax and polyphenol layers before reaching the pore canals (assuming that the pore canals penetrate the cuticulin layer). In *Ornithodorus* water is probably taken up through the cement layer as well.

The tick epicuticle, like that of insects (Wigglesworth, 1947), is evidently a complex laminated structure. In the family Argasidae there are four components comprising the cuticulin, polyphenol, wax and cement layers. In the Ixodidae only the three inner layers are represented, the outermost covering of cement being absent. A further difference concerns the type of wax secreted. Without exception the waxes of the argasid group have higher critical temperatures and confer a more perfect degree of impermeability than the ixodid waxes. No doubt both the structure of the epicuticle and the nature of the wax will influence the ease of penetration through the cuticle of such substances as insecticidal materials. The simultaneous presence of a high melting point wax and a cement covering in *Ornithodorus* may account for the resistance of this species to nearly all contact insecticides and to injurious solvents, such as xylenol (Robinson, 1942). On the other hand, as is well known, the Ixodidae are relatively susceptible to insecticides, even to aqueous solutions of poisons such as sodium arsenite (Burtt, 1945).

The deposition of the different layers in the epicuticle follows the general pattern described in *Rhodnius* (Wigglesworth, 1947). The cuticulin and polyphenol layers are secreted first, the wax afterwards. There is a difference in timing, however. The wax layer is laid down precociously in ticks, deposition being well advanced by the time moulting fluid becomes abundant. All three layers are secreted by the epidermal cells. In Ixodidae the dermal glands appear to contribute nothing of functional significance to the structure of the cuticle. In *Ornithodorus moubata*, on the other hand, the cement is probably secreted by the dermal glands and is poured over the surface of the wax layer shortly after emergence. The general parallelism between the structure and development of the epicuticles in *Ornithodorus* and *Rhodnius* is remarkable: the identity of the epicuticular layers, their mode of deposition, and even details such as the delayed deposition of wax over the muscle insertions, are very similar. That there are differences in the chemistry of the outermost layers is suggested, however, by the lack of effect in *Ornithodorus* of detergents which in *Rhodnius* are very efficient in attacking the cement and wax layers.

SUMMARY

1. Ticks owe their impermeability primarily to a superficial layer of wax in the epicuticle. After exposure to increasing temperatures, water loss increases abruptly at a certain 'critical temperature'. The critical temperature varies widely in different species, in Ixodidae ranging from 32 (*Ixodes ricinus*) to 45° C. (*Hyalomma savignyi*); and in Argasidae from 63 (*Ornithodorus moubata*) to 75° C. (*O. savignyi*). Species having higher critical temperatures are more resistant to desiccation at temperatures within the biological range. A broad correlation is possible between these powers of resistance and the natural choice of habitat. Argasidae infest dry, dusty situations whereas Ixodidae occupy a much wider variety of 'ecological niches'.

2. If the tick cuticle is rubbed with abrasive dust, evaporation is enormously increased. Living ticks partially restore their impermeability in moist air by secreting wax from the pore canals on to the surface of the damaged cuticle.

3. Unfed ticks are able to take up water rapidly through the wax layer when exposed to high humidities. Water uptake, which is dependent on the secretory activities of the epidermal cells, is completely inhibited by the abrasion of only part of the total cuticle surface—a fact which suggests that the cells are functionally interconnected. Resistance to desiccation at low humidities is achieved by a dual mechanism: active secretion and the physical retention of water by the wax layer.

4. In Argasidae the epicuticle consists of four layers: the cuticulin, polyphenol, wax and outer cement layers. Only the three inner layers are present in Ixodidae. Since the wax layer is freely exposed in the latter group, chloroform and detergents have a marked action in increasing transpiration, particularly in those species with low critical temperatures. In Argasidae the cement layer is very resistant to extraction but is broken down by boiling chloroform.

5. The cuticulin, polyphenol and wax layers are all secreted by the epidermal cells. The waterproofing layer, which is deposited on the completed polyphenol layer, is secreted by the moulting tick relatively early in development and may be nearly complete by the time moulting fluid is abundant. In *Ornithodorus moubata* the cement is poured out by the dermal glands shortly after emergence. In Ixodidae the dermal glands undergo a complex cycle of growth and degeneration, but their products appear to add nothing of functional significance to the substance of the cuticle.

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